FUGETACTIC PROTEINS, COMPOSITIONS AND METHODS OF USE

Government Support

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Related Applications/Patents & Incorportaion By Reference

This application claims priority to U.S. Application Serial No. 60/485,550 filed July 7, 2003.

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Field of the Invention

This invention relates to compositions and methods that modulate the movement of cells with migratory capacity. More specifically, the invention relates to compositions and methods for promoting migratory movement of cells away from a specific site in a subject (i.e., fugetaxis) or alternatively inhibiting such movement. The foregoing are useful, *inter alia,* in the treatment of conditions that would benefit from such migratory cell movement.

Background of the Invention

Cell movement in response to specific stimuli is known to occur in prokaryotes and eukaryotes (Doetsch RN and Seymour WF.,1970; Bailey GB et al.,1985). The cell movements seen in these organisms has traditionally been classified into three types; chemotaxis or the movement of cells along a gradient towards an increasing concentration of a chemical; negative chemotaxis (or "fugetaxis") which has been described as the movement down a gradient of a chemical stimulus and chemokinesis or the increased random movement of cells induced by a chemical agent. The receptors and signal transduction pathways for the actions of specific chemotactically active compounds have been extensively defined in prokaryotic cells. Study of *E. coli* chemotaxis has revealed that a chemical which attracts the bacteria at some concentrations and conditions may also induce negative chemotaxis or "fugetaxis" at others (Tsang N et al., 1973; Repaske D and Adler J. 1981; Tisa LS and Adler J.,1995; Taylor BL and Johnson MS., 1998).

Identification and characterization of eukaryotic fugetactic polypeptides, and inhibitors thereof, could lead to the development of therapeutic agents having a specialized activity that is useful for regulating migratory cell movement.

Summary of the Invention

As demonstrated herein fugetactic agents have been discovered that function to repel migratory cells away from a specific site. In certain aspects of the invention these fugetactic agents and compositions thereof are provided. In some embodiments the compositions provided herein are in effective amounts to promote fugetaxis. In other aspects of the invention anti-fugetactic agents are provided, and the compositions which comprise these anti-fugetactic agents can also be in effective amounts to inhibit fugetaxis. In other aspects of the invention methods of use of the compositions provided herein are given.

In some instances the fugetactic agents provided are heat shock proteins (HSPs). In other instances these fugetactic agents are heat shock protein-like proteins (HSPLPs). In some aspects of the invention the HSPs or HSPLPs are combined with a pharmaceutically acceptable carrier. In some aspects the HSP or HSPLP is in an effective amount to promote fugetactic activity. In some

embodiments the HSP or HSPLP has a molecular weight of about 84 kDa. In other embodiments the HSP or HSPLP has a molecular weight of about 86 kDa. In still other embodiments the HSP or HSPLP has a molecular weight of about 94 kDa.

In yet other embodiments the HSPs or HSPLPs provided are members of an HSP family such as HSP60 (chaperonin), HSP70, or HSP90 families. In specific embodiments the HSP or HSPLP is a member of the hsp90 family. In some embodiments the HSP or HSPLP is HSP 90α or is HSP 90β, or variants thereof. The HSP or HSPLP proteins provided herein therefore encompass proteins encoded by amino acid sequences as set forth as SEQ ID NOs: 1-8, as well as fragments and variants thereof (Fig. 1)

HSP or HSPLP proteins provided herein also include proteins which contain any of the amino acid sequences provided herein or combinations thereof. In some embodiments the amino acid sequences are given in Figs. 12 and 13 (Table 1). In some embodiments the amino acid sequences are those represented in Figs. 12 and 13 (Table 1), but without either or both of the amino acid residue(s) located in each end position.

In still other embodiments the HSPs or HSPLPs are in a secreted form. In these embodiments the HSPs or HSPLPs in secreted form can contain a signal sequence or a secretory sequence. In still other embodiments the HSPs or HSPLPs are not in a secreted form. In still further embodiments the HSPs or HSPLPs contain a retention signal.

The HSPs or HSPLPs can be obtained from any cell that produces, or can be manufactured to produce, the HSPs or HSPLPs described herein. For instance, in some embodiments the HSPs or HSPLPs are from stressed or non-stressed cells. In other embodiments the cells are stromal cells. In yet other embodiments the cells are thymic stromal cells. In still other embodiments the HSPs or HSPLPs are from a tumor cell or a tumor cell line. The tumor or tumor cell line can be derived from any kind of tumor. In some embodiments the tumor or tumor cell line is a hematological tumor or a hematological tumor cell line. In yet other embodiments the hematological tumor or hematological tumor cell line is a leukemia or a lymphoma. In other embodiments the hematological tumor or hematological tumor cell line is a thymoma cell or thymoma cell line. In certain of these embodiments the thymoma cell line is EL4.

In addition to HSPs and HSPLPs, which are now found to have fugetactic activity, L-plastin has also been found to exhibit similar function. Therefore, in some aspects of the invention compositions of L-plastin which have fugetactic activity are provided. In still other aspects of the invention L-plastin-like proteins (LPLPs) with fugetactic activity are provided. Therefore, in one aspect, a pharmaceutical composition that contains an isolated L-plastin or L-plastin-like protein in an effective amount to promote fugetactic activity and a pharmaceutically acceptable carrier is provided. In some embodiments the L-plastin or LPLP has a molecular weight of about 65 kDa. In still other embodiments the L-plastin or LPLP comprises an amino acid sequence such as SEQ ID NO: 8 (Fig. 1). The L-plastin or LPLP proteins provided herein also include proteins which contain any of the amino acid sequences provided herein or combinations thereof. In some embodiments the amino acid sequences are given in Fig. 14 (Table 1). In some embodiments the amino acid sequences are those represented in Fig. 14, but without either or both of the amino acid residue(s) located in each end position.

In some embodiments the L-plastin or LPLP, provided herein, is in a secreted form. In some embodiments the secreted form of the L-plastin or LPLP comprises a signal sequence or a secretory sequence. In still other embodiments the L-plastin or LPLP can be in phosphorylated or unphosphorylated form

L-plastin or LPLP can also be obtained from any cell that produces or is made to produce the protein. These cells include in some embodiments stromal cells such as thymic stromal cells. These cells in other embodiments include tumor cells or tumor cell lines. In other embodiments the tumor or tumor cell line is a hematological tumor or a hematological tumor cell line. In still other embodiments the hematological tumor or hematological tumor cell line is a leukemia or a lymphoma. In some of these embodiments the lymphoma is a thymoma. In yet other embodiments the tumor cell line is EL4.

The fugetactic agents provided herein also include cell isolates. Therefore, in one aspect of the invention a pharmaceutical composition containing an isolate as described herein in an effective amount to stimulate fugetaxis of a cell, and a pharmaceutically acceptable carrier is provided. The cell isolates can be from any cell such as a thymic stromal, tumor or tumor cell line. In some embodiments the tumor cell line can be a thymoma cell line. In some of these embodiments the

thymoma cell line is EL4. Cell isolates as provided herein include any material obtained from or including a cell, which can exhibit fugetactic activity. Cell isolates therefore include supernatants, fractions, diluted supernatants and fractions and molecules (e.g. polypeptides). In some embodiments the isolate is a substantially pure polypeptide. In still other embodiments the isolate is a supernatant of the EL4 thymoma cells. In yet other embodiments the isolate is a diluted supernatant. In some of these embodiments the supernatant is diluted ten-fold.

In one aspects the cell isolate is an isolate from a thymoma cell line, wherein the isolate has fugetactic activity that is pertussis toxin inhibitable, protease degradable, and has a molecular weight of greater than about 5 kDa and is heat inactivatable. In some embodiments the fugetactic activity of the isolate can be inhibited by heat inactivation at 56°C for one hour. In other embodiments the fugetactic activity of isolate can be inhibited by proteinase K digestion at 37°C for one hour. In still other embodiments the isolate does not bind significantly to heparin. In yet other embodiments the isolate binds significantly to a DEAE column in the presence of 20mM triethanolamine buffer and NaCl in a concentration lower than 0.25-0.5M. In still further embodiments the isolate is negatively charged at pH 7.5. In yet other embodiments the fugetactic activity of the isolate can be inhibited by radicicol. In yet other embodiments the fugetactic activity of the isolate is inhibited by Geldanamycin. In still other embodiments the production of the isolate by the thymoma cells can be inhibited by Brefeldin A. In still further embodiments the activity of the isolate is not significantly upregulated by heat shock at 42°C for one hour. In certain embodiments the isolates are obtained from cells that are not undergoing significant apoptosis or necrosis. In some embodiments the isolates are obtained from a cell (e.g. a thymoma cell line such as EL4) that is greater than 95% viable.

The isolate in some embodiments has a molecular weight that is greater than about 65 kDa. In other embodiments the isolate has a molecular weight that is greater than about 80 kDa. In still other embodiments the cell isolate has a molecular weight that is greater than about 90 kDa.

In another aspect of the invention a method of promoting fugetaxis of migratory cells in a subject is provided. This method includes administering to a subject in need of such treatment a HSP, HSPLP, L-plastin or LPLP provided

herein, in an amount effective to promote fugetaxis of migratory cells away from a specific site in a subject. In some embodiments the method further includes co-administering a non-fugetactic therapeutic agent. In some embodiments the non-fugetactic agent is an anti-inflammatory or an anti-allergic agent.

The migratory cells as taught herein can be any cell with migratory capacity. In some embodiments the migratory cells are hematopoietic cells. In some of these embodiments the hematopoietic cells are immune cells. In still other embodiments the immune cells are T cells. In yet other embodiments the migratory cells are cytotoxic T lymphocytes (CTLs).

The specific site of this method can be any site where the movement of migratory cells is needed. For instance, in some embodiments the specific site is a site of an inflammation. In some embodiments the specific site is a site of an autoimmune reaction. In some of these embodiments the site of an autoimmune reaction is a site at or near a joint. In yet other embodiments the specific site is a site of an allergic reaction. In still other embodiments the specific site is a medical device, prosthetic device or a transplanted organ or tissue. In some of these embodiments the medical device, prosthetic device or a transplanted organ or tissue is xenogeneic, stem-cell derived, synthetic or an allograft. In other embodiments the medical device, prosthetic device or a transplanted organ or tissue is a stent.

The compositions provided herein can be administered in any way that is effective. In some embodiments the composition is administered locally. In other embodiments the composition is administered systemically. The compositions provided herein can be administered therefore to treat local or systemic conditions. In still other embodiments the compositions can be targeted to a specific site. For instance, in some embodiments the HSP, HSPLP, L-plastin or LPLP provided herein is conjugated to a targeting molecule.

In addition to fugetactic agents, anti-fugetactic agents are also provided. In one aspect of the invention a pharmaceutical composition that contains an anti-fugetactic agent that selectively binds to any of the HSPs, HSPLPs, L-plastins or LPLPs as provided herein in an effective amount to inhibit fugetactic activity and a pharmaceutically acceptable carrier is provided. The anti-fugetactic agent can bind to any of the amino acid sequences provided herein or a portion thereof; and

therefore, can bind in some aspects to proteins which comprise these amino acid sequences.

The anti-fugetactic agents provided can be any of a number of molecules that are able to selectively bind to the fugetactic agents provided and/or inhibit fugetaxis. In some embodiments the anti-fugetactic agent is an isolated peptide. In still other embodiments the anti-fugetactic agent is an antibody or an antigen-binding fragment thereof. In yet other embodiments the anti-fugetactic agent is a small molecule.

Included herein in one aspect is a method of eliciting or enhancing an immune response in a subject. This method includes administering to a subject in need of such treatment an anti-fugetactic agent provided herein in an amount effective to inhibit immune cell-specific fugetactic activity at a specific site in the subject. The method covers any site for which the inhibition of fugetaxis would be beneficial. In some embodiments the specific site is a site of an infection. In other embodiments specific site is a tumor.

The methods provided herein further cover the use of any anti-fugetactic agent. In some embodiments the anti-fugetactic agent is Geldanamycin, 17-A-GA, herbimycin A, PU3, novobiocin or radicicol. In other embodiments the anti-fugetactic agent is an agent other than a benzoquinoid ansamycin. In still other embodiments the anti-fugetactic agent is an agent other than Geldanamycin, 17-A-GA, herbimycin A, PU3, novobiocin or radicicol.

In another aspect of the invention a method of screening for an anti-fugetactic agent that modulates fugetaxis is provided. This method contains the steps of determining a control level of fugetactic activity by combining a migratory cell with an HSP, HSPLP, L-plastin or LPLP, determining a test level of fugetactic activity by combining a migratory cell with the HSP, HSPLP, L-plastin or LPLP and a candidate compound, and comparing the control and test levels of the fugetactic activity, wherein a test level that is less than a control level indicates that the candidate compound is an anti-fugetactic agent. The fugetactic agent used in the screening method can be any of the fugetactic agents described herein.

In another aspect of the invention a method of producing a polypeptide having fugetactic activity from cells (e.g. thymoma cell such as EL4 tumor cells) by culturing the cells at a density of 10^5-10^6 cells/mL in hybridoma serum free medium, harvesting a supernatant from the cells, filtering the harvested supernatant with a 0.2

micron filter, fractionating the filtered supernatant, and analyzing the fractions for fugetactic activity are provided. In some embodiments the cultured cells are greater than 95% viable.

In other aspects the polypeptide having fugetactic activity produced according to the method is also provided. In some embodiments the polypeptide has a molecular weight of about 84 kDa. In still other embodiments the polypeptide has a molecular weight of about 86 kDa. In yet other embodiments the polypeptide has a molecular weight of about 94 kDa. In still further embodiments the polypeptide has a molecular weight of about 65 kDa.

In still other aspects of the invention a pharmaceutical composition that contains the polypeptide produced in an effective amount to stimulate fugetaxis of cells, and a pharmaceutically acceptable carrier is provided.

In still a further aspect of the invention a method of screening for an anti-fugetactic agent that modulates fugetaxis, which includes the steps of determining a control level of fugetactic activity by combining a migratory cell with any isolate or polypeptide provided herein; determining a test level of fugetactic activity by combining a migratory cell with the isolate or the polypeptide, and a candidate compound; and comparing the control and test levels of the fugetactic activity, wherein a test level that is less than a control level indicates that the candidate compound is an anti-fugetactic agent is provided.

Another aspect of the invention is a method of promoting fugetaxis of cells in a subject, which includes the steps of administering to a subject in need of such treatment an isolate or the polypeptide provided herein, in an amount effective to promote fugetaxis of cells away from the specific site in the subject. In some embodiments the specific site is a site of inflammation. In still other embodiments the specific site is a medical device, prosthetic device or transplanted organ or tissue. In yet other embodiments the specific site is a site of an autoimmune reaction. In still other embodiments the specific site is a site of an allergic reaction.

The methods provided herein can further include in some embodiments co-administering a non-fugetactic therapeutic agent. In some embodiments the non-fugetactic therapeutic agent is an anti-inflammatory or an anti-allergic agent. Other non-fugetactic therapeutic agents are anti-cancer agents. In some other

embodiments methods are provided which can further include the administration of a second fugetactic or anti-fugetactic agent.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments.

Brief Description of the Drawings

Fig. 1 describes the amino acid sequences of HSP 90, HSP 84, HSP 86, HSP 60 and L-plastin.

Fig. 2 provides the results of a transmigration assay using 1 in 2, 1 in 10 and 1 in 100 dilutions of EL4 24-hour conditioned media (EL4CM24).

Fig. 3 provides the results of a transmigration assay using negative gradients of heat inactivated or proteinase K digested EL4 24-hour conditioned media (EL4CM24) (1 in 2, 1 in 10 and 1 in 100 dilutions).

Fig. 4 provides the results of a transmigration assay using negative gradients of EL4 24-hour conditioned media (EL4CM24) with pertussis toxin treated murine lymphocytes and radicicol and Geldanamycin treated EL4CM24 (1 in 2, 1 in 10 and 1 in 100 dilutions).

Fig. 5 provides the results of an *in vivo* study of the migration of immune cells using EL4 24-hour conditioned media (EL4CM24).

Fig. 6 provides the results of EL4 24-hour conditioned media (EL4CM24) (I0.5 and HSF) run on SDS PAGE.

Fig. 7 provides the results of the ion exchange chromatography of the EL4 24-hour conditioned media (EL4CM24).

Fig. 8 provides the results of a transmigration assay using EL4 24-hour conditioned media (EL4CM24) heat shocked at 42°C and treated with Brefeldin A.

Fig. 9 provides the mass peaks from the mass spectrometry analysis of a fraction of EL4 24-hour conditioned media (EL4CM24) that contained a protein of about 84/86 kDa.

Fig. 10 provides the mass peaks from the mass spectrometry analysis of a fraction of EL4 24-hour conditioned media (EL4CM24) that contained a protein of about 94 kDa.

Fig. 11 provides the mass peaks from the mass spectrometry analysis of a fraction of EL4 24-hour conditioned media (EL4CM24) that contained a protein of about 65 kDa.

Fig. 12 provides the MS-Fit and MS-Tag search results of a component protein of about 84 and 86 kDa.

Fig. 13 provides the MS-Fit search results of a component protein of about 94 kDa.

Fig. 14 provides the MS-Fit and MS-Tag search results of a component protein of about 65 kDa.

Fig. 15 provides the sequence alignment of human HSP 90-β and mouse HSP protein 84.

Fig. 16 provides the sequence alignment of HSP 84 and HSP 86, both from the mouse.

Detailed Description of the Invention

It has now been discovered, according to the invention, that tumor cells elaborate both chemokines and other chemokinetically active substances which evoke a fugetactic or chemorepellent response from immune cells, thereby allowing the neoplastic cells to evade recognition and destruction by the host immune system. Using *in vitro* and *in vivo* assays it has now been demonstrated that culture supernatant (i.e., conditioned media) from the EL4 cell line has the ability to repel lymphocytes (i.e., to induce fugetaxis). It has been further shown that migration of lymphocytes away from EL4 24-hour conditioned media (EL4CM24) was diminished by heat inactivation and proteinase digestion of the conditioned media as well as with the use of the specific inhibitors (pertussis toxin and radicicol). Fractionation and subsequent tests on the conditioned media fractions resulted in the identification of agents which induce fugetaxis. Some of these agents show homology to heat shock proteins (HSPs) as well as L-plastin.

The present disclosure therefore provides, in part, agents with migratory cell repellent activity (hereinafter "fugetactic agents" and "fugetactic activity" or

"fugetaxis"). Such agents include tumor cell isolates, heat shock proteins and L-plastin, which have now been discovered to possess fugetactic activity.

HSPs are proteins that have been found to be monoallelic and virtually intracellular (Srivastava, P., Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu. Rev. Immunol.* 2002. 20:395-425.) Surprisingly, this is the first demonstration that HSPs exist extracellularly and more importantly that they possess fugetactic activity. Prior to the present disclosure, HSPs were thought to be found extracellularly only as a result of necrosis. HSPs are involved in stress response, protein folding, protein degradation, control of mutant expression, regulation of transcription, differentiation, cell death, and chaperoning a wide array of peptides, such as antigenic peptides. HSPs are further thought to be involved in immune responses through the stimulation of inflammatory cytokine secretion, involvement in the MHC I and II pathways and the mediation of immune cell maturation (Srivastava, P., et al., Heat shock proteins: the 'Swiss Army Knife' vaccines against cancers and infectious agents. *Vaccine.* 19 (2001). 2590-2597.) Fugetactic activity of HSPs has not yet been described. Not bound by any particular theory, it is thought that the fugetactic activity of HSPs could be through the direct fugetactic activity of these proteins or their indirect action through the presentment or sequestration of fugetactic cytokines or chemokines.

Although, HSPs were originally recognized in cells that were heat-shocked, HSPs are also expressed in cells under normal conditions. However, it is commonly observed that under stressed conditions (e.g., heat exposure, toxin exposure, glucose deprivation, etc.), HSPs levels can be highly induced (Srivastava, P., Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu. Rev. Immunol.* 2002. 20:395-425.) Examples of these proteins, just to name a few, include HSP 27kDa protein (Accession No. NP_001532), HSP 70 kDa protein (Accession No. P08107), HSP 60 kDa protein (chaperonin) (Accession No. NP_002147), cardiovascular HSP (Accession No. CAB86671), HSP apg-1 (Accession No. AAp44471) and HSP (110 family) (Accession No. NP_055093). Other heat shock proteins are well known in the art or can be easily discovered. Not only are HSP members within HSP families quite homologous, but HSPs between species have also been found to exhibit

significant homology. For instance, rat ischemia-responsive protein 94 kDa (irp94), which is a member of the HSP110 family, was found to be greater than 90% identical to mouse heat shock protein 4, apg-2 (Accession No. AAH03770) and human HSP70RY (Accession No. NP_705893) (Yagita, Y., et al., Molecular cloning of a novel member of the HSP110 family of genes, ischemia-responsive protein 94 kDa (irp94), expressed in rat brain after transient forebrain ischemia. *J. Neurochem.* 1999. 72(4): 1544-51.) Additionally, it was found that irp94 was greater than 60% identical to other HSP110 family members. A homology study presented in Fig. 15 shows that human HSP90β and mouse HSP84 are approximately 85% identical. Mouse HSPs 84 and 86 have also shown significant homology (Fig. 16). An analysis of these sequences showed that they are approximately 76% identical.

HSPs are intended to encompass proteins that belong to any of the HSP families. Examples of HSP families include, but are not limited to, the HSP60 (chaperonin), HSP70, and HSP90 families. In specific embodiments, HSPs of the invention include SEQ ID NOs: 1-7 (Fig. 1). HSPs may also simply be variants of HSPs that contain a secretory or signal sequence that facilitates their secretion outside a cell. In a specific embodiment, the HSP is a secreted variant of HSP 90β. HSPs also encompass HSPs that have a retention signal.

The fugetactic agents provided herein may also be heat shock protein-like proteins (HSPLPs). HSPLPs are proteins with homology to heat shock proteins (HSPs) or that exhibit similar structural characteristics and functions as heat shock proteins. These functions include functions traditionally associated with HSPs but also include functions, such as fugetactic activity, as provided herein. These proteins may be secreted from tumor or other cells.

The fugetactic agents, provided herein, also include L-plastin proteins. One of the proteins that has now been identified to exhibit fugetactic activity is a protein that is approximately 65kDa and exhibits homology to L-plastin. L-plastin is a polypeptide found in cells of the hematopoietic system (Shinomiya, H., et al., Complete primary structure and phosphorylation site of the 65-kDa macrophage protein phosphorylated by stimulation with bacterial lipopolysaccharide. *The Journal of Immunology*. 1995. 154: 3471-3478.) Because the function of L-plastin can be modulated by phosphorylation, the L-plastins described herein can be in phosphorylated or unphosphorylated form. L-plastin proteins, like HSPs, are quite

homologous. The murine and human forms of L-plastin have been found to be greater than 95% identical (Shinomiya, H., et al., Complete primary structure and phosphorylation site of the 65-kDa macrophage protein phosphorylated by stimulation with bacterial lipopolysaccharide. *The Journal of Immunology*. 1995. 154: 3471-3478. Also L-plastin has been found to have Ca2+ binding sites as well as an actin binding domain. In fact, L-plastin has also been found to act on β-actin in a Ca^{2+}-dependent fashion. L-plastin is also thought to have a calmodulin binding site.

Fugetactic agents also include L-plastin like proteins (LPLPs). Such proteins are proteins that exhibit homology to L-plastin (Fig. 1, SEQ ID NO:8) or proteins that exhibit structural and functional characteristics similar to L-plastin.

Homology between proteins can be determined in terms of the amino acid sequence or nucleic acid sequence of the protein. In certain embodiments, the amino acid sequences of homologous proteins are greater than about 30% identical. In other embodiments, the homologous proteins have amino acid sequences that are greater than about 40%, 50%, 60%, 70%, 80%, 90% or 95% identical. In yet other embodiments, the homologous proteins are encoded by nucleic acid molecules that are at least about 10% identical. In still other embodiments the nucleic acid molecules are at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% identical. In still other embodiments the nucleic acid molecules are at least about 95% or 99% identical. Homology can be calculated using various, publicly available software tools well known to one of ordinary skill in the art. Exemplary tools include the BLAST system available from the website of the National Center for Biotechnology Information (NCBI) at the National Institutes of Health.

Another method of identifying homologous nucleotide sequences is via nucleic acid hybridization. In some embodiments, the nucleic acid hybridization is carried out under high stringency conditions. Identification of related sequences can also be achieved using polymerase chain reaction (PCR) and other amplification techniques suitable for cloning related nucleic acid sequences.

The term "high stringency conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references that compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory

Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. One example of high-stringency conditions is hybridization at 65°C in hybridization buffer (3.5X SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH_2PO_4(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.015M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, a membrane upon which the nucleic acid is transferred is washed, for example, in 2X SSC at room temperature and then at 0.1 - 0.5X SSC/0.1X SDS at temperatures up to 68°C.

The fugetactic agents provided also include tumor cell isolates, which may be derived from tumor cells or cell lines or from the culture supernatant in which such cells are grown. The fugetaxis-promoting tumor cell isolates can be derived from any tumor cell or cell line. A "tumor cell isolate" is any material that contains or originates from a tumor cell or cell line. The tumor cell isolate, therefore, includes supernatants, fractions of the supernatants, diluted supernatants and fractions as well as isolated molecules (e.g. polypeptides) from the tumor cell or cell line. The tumor cell isolates can be derived from thymoma cells, such as EL4 thymoma cells, but they are not so limited. Tumor cell isolates can be derived from any thymoma cell line. Such thymoma cell lines include BW5147.3 (ATCC No. TIB-47), BW5147.G.1.4 (ATCC No. TIB-48), WEHI 7.1 (ATCC No. TIB-53), WEHI 22.1 (ATCC No. TIB-54). Tumor cell isolates can also be derived from any tumor cell or tumor cell line. For example, these include cells of a melanoma, lymphoma, T cell lymphoma, B cell lymphoma, hepatoma, sarcoma, fibrosarcoma or mastocytoma. Other examples of tumor cells are provided herein below. In some embodiments, these tumors or tumor cell lines have been found to express HSPs and L-plastin. In other instances, the tumor cells are non-stressed or stressed cells. In still other instances, the cells from which the fugetactic agents are obtained are not undergoing significant apoptosis or necrosis. In still further, embodiments these cells are greater than 95% viable.

The fugetactic agents provided also include stromal-cell or stromal cell line isolate. Stromal cells are found in loose connective tissue. Stromal-cells may readily be derived from organs, that include, but are not limited to, skin, liver, pancreas, bone marrow, lymph node, thymus, kidney, CNS, brain, etc., using

methods known in the art such as those discussed above. Such cells include, but are not limited to, endothelial cells, pericytes, macrophages, monocytes, plasma cells, mast cells, adipocytes, etc. In one example, stromal cells of hematopoietic tissue, including but not limited to, fibroblasts, endothelial cells, macrophages/monocytes, adipocytes and reticular cells, could be used to inoculate a tissue culture dish and/or a three-dimensional matrix. In some embodiments the isolates are thymic stromal cell isolates derived from thymic stromal cells or thymic stromal cell lines.

Thus, the invention provides fugetactic agents from tumor cells or cell lines. Preferably, these fugetactic agents are secreted from the tumor cells or cell lines, and thus can be obtained from the culture supernatant of these cells. Alternatively, the fugetactic agents may also be obtained from the cells themselves, presumably in a membrane bound form or an intracellular form.

The invention further provides pharmaceutical compositions and methods of use for these fugetactic agents (e.g., the tumor cell isolates, HSPs, HSPLPs, L-plastin and LPLPs). Pharmaceutical compositions and methods of use for inhibitors of these fugetactic agents are also provided by the invention. The foregoing can be used, *inter alia,* in the treatment of conditions characterized by inappropriate migratory cell movement from specific sites in a subject.

"Fugetactic activity" or "fugetaxis" refers to the ability of an agent to repel (or chemorepel) a eukaryotic cell with migratory capacity (i.e., a cell that can move away from or towards a stimulus). Accordingly, an agent with fugetactic activity is a "fugetactic agent." Such activity can be detected and measured using any of the transmigration systems described herein (see Examples), or a variety of other systems known in the art (see, e.g., U.S. Pat. No. 5,514,555, entitled: "Assays and Therapeutic Methods Based on Lymphocyte Chemoattractants" issued May 7, 1996, to Springer, TA, et al.).

The fugetactic agents of the invention are capable of repelling cells with migratory capacity and in particular hematopoietic cells, such as immune cells. Even more particularly the migratory cells are T cells. The fugetactic agents of the invention are useful in conditions where inhibition of migration of immune cells to a specific site is desirable. Many of these conditions are associated with an inflammatory response. Inhibitors of the fugetactic agents of the invention are conversely useful in conditions where promotion of migration of immune cells to a

specific site is desirable. These latter conditions include but are not limited to tumors and infections. Preferably, the fugetactic agents of the invention are peptides or proteins.

The fugetactic agents of the invention can have a molecular weight of about 50 kDa, 55 kDa, 60 kDa, 65 kDa, 70 kDa, 75 kDa, 80 kDa, 85 kDa, 90 kDa, 95 kDa, 100 kDa, 105 kDa, 110 kDa, 115 kDa, 120 kDa, 125 kDa, 130 kDa, 135 kDa, 140 kDa, 145 kDa or 150 kDa, etc. In some instances the fugetactic agent has a molecular weight of about 65 kDa, 84 kDa, or about 86 kDa or about 94 kDa.

Proteins or polypeptides of the invention, are optionally recombinant, including whole proteins, partial proteins (e.g., domains) and peptide fragments. Fragments of a polypeptide preferably are those fragments that retain the distinct functional capability of the particular protein, which in this case is fugetactic activity. Such polypeptides can also comprise, for example, fusion proteins and chimeric proteins. Short polypeptides can be synthesized chemically using well-established methods of peptide synthesis.

The invention also contemplates the use of HSP, HSPLP, L-plastin and LPLP variants. As used herein, a "variant" of a polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence, including truncations and deletions. Variants would include allelic variants and polymorphic variants having conserved function. Modifications which create a polypeptide variant can also be made to 1) enhance a property of a polypeptide, such as protein stability in an expression system or the stability of protein-protein binding; or 2) to provide a novel activity or property to a polypeptide, such as addition of a detectable moiety. Modifications to a polypeptide can be introduced by way of the nucleic acid which encodes the polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the amino acid sequence.

The skilled artisan will also realize that conservative amino acid substitutions may be made in the neuronal differentiation factors described above to provide functionally equivalent variants of the foregoing polypeptides, i.e., the variants

retaining functional capabilities. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York.

Conservative amino-acid substitutions in amino acid sequences typically are made by alteration of the coding nucleic acid encoding. Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a coding gene. Where amino acid substitutions are made to a small peptide fragment, the substitutions can be made by directly synthesizing the peptide. The activity of functionally equivalent fragments of polypeptides can be tested by cloning the gene encoding the altered polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered polypeptide, and testing for a functional capability. Peptides which are chemically synthesized can be tested directly for function.

As stated above, fugetactic agents include tumor cell isolates, e.g. a thymoma cell isolate, with fugetactic activity. The tumor cell isolate may be derived from any tumor type. In one embodiment, the isolate is derived from the culture supernatant of a thymoma cell, such as the EL4 thymoma cell line. The tumor cell isolate preferably is protease sensitive and heat inactivatable. In other embodiments the fugetactic activity of the isolate can be inhibited by heat inactivation at 56°C for one hour. In yet other embodiments, the fugetactic activity of the isolate can be inhibited by proteinase K digestion at 37°C for one hour. In still other embodiments, the fugetactic activity of the isolate can be inhibited by pertussis toxin treatment. In still other embodiments, the fugetactic activity of the isolate can be inhibited by radicicol. In yet other embodiments, the fugetactic activity of the isolate can be

inhibited by Geldanamycin. Further in some instances, the isolate does not bind significantly to heparin. In yet other instances, the isolate binds significantly to a DEAE column in the presence of 20 mM triethanolamine buffer and NaCl in a concentration lower than 0.25-0.5 M. In still further embodiments, the isolate is negatively charged at pH 7.5. In other embodiments, the production of the isolate by the thymoma cells can be inhibited by Brefeldin A. In still further embodiments, inhibition by Brefeldin A is produced by Brefeldin A treatment at 10 μg/mL final concentration. In another embodiment the activity of the isolate is not significantly upregulated by heat shock at 42°C for one hour. In further particular embodiments, the cell isolate from the EL4 cell line has a molecular weight greater than about 5 kDa. The cell isolate can also have a molecular weight greater than about 20 kDa, 30 kDa, 40 kDa, 50 kDa, 60 kDa, 70 kDa, 80 kDa, 90 kDa, 100 kDa, 110 kDa, 120 kDa, 130 kDa, etc. More specifically, the cell isolate can have a molecular weight of about 60 kDa, 65 kDa, 70 kDa, 75 kDa, 80 kDa, 85 kDa, 90 kDa, 95 kDa, 100 kDa, 105 kDa, 110 kDa, 115 kDa, 120 kDa, 125 kDa, 130 kDa, 135 kDa, 140 kDa, 145 kDa or 150 kDa, etc. Even more specifically, the cell isolate has a molecular weight of about 65 kDa, 84 kDa, 86 kDa or 94 kDa. In preferred embodiments, the cell isolate comprises a polypeptide, and it is this polypeptide that repels immune cells. In some instances, this polypeptide is an HSP, HSPLP, L-plastin or LPLP, and it is this HSP, HSPLP, L-plastin or LPLP that repels immune cells.

As used herein with respect to tumor cell isolates, "isolate (i.e. isolated)" means separated from its native environment and present in sufficient quantity to permit its identification or use. Isolated, when referring to a protein or polypeptide, means, for example: (i) selectively produced by expression cloning or (ii) partially purified as by chromatography or electrophoresis. Isolated proteins or polypeptides may, but need not be, substantially pure. In certain embodiments of the present invention the thymoma cell isolate is a substantially pure polypeptide. The term "substantially pure" means that the protein(s) or polypeptide(s) is essentially free of other substances with which it (they) may be found in nature or *in vitro* systems, to an extent practical and appropriate for their intended use. Substantially pure polypeptides may be produced by techniques well known in the art. Because an isolated protein may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the protein may comprise only a small percentage by

weight of the preparation. The protein is nonetheless isolated in that it has been separated from many of the substances with which it may be associated in living systems, i.e. isolated from certain other proteins.

As stated above, the fugetactic agents include HSPs and L-plastin. These proteins have been discovered to possess fugetactic activity, according to the invention. It has been discovered that eluted fractions of an EL4 24-hour conditioned medium (EL4CM24) comprise proteins having amino acid sequences in common with HSPs and L-plastin. These amino acid sequences are provided in Figs. 12-14. Therefore, the fugetactic agents provided herein include proteins, which include the amino acid sequences depicted in Figs. 12-14 (set forth as SEQ ID NOs:9-118 , which include both end residues). Fugetactic agents also include proteins which comprise a portion of these sequences (e.g. the amino acid sequences set forth as SEQ ID NOs:9A-118A which are the same as SEQ ID NOs:9-118 but lack either or both of the end residues, which are denoted by parenthesis). The fugetactic agents of the invention, therefore, can comprise an amino acid sequence that is any one of SEQ ID NOs:1-8. The fugetactic agents of the invention can be HSP90α (Fig. 1, SEQ ID NO:3), HSP90β (Fig. 1, SEQ ID NOs:1 and 2) or L-plastin (Fig. 1, SEQ ID NO:8).

Table 1

Peptide Sequence	Fig. No.
(K)VTISNR(L) SEQ ID NO:9, 9A	12
(R)ALLFIPR(R) SEQ ID NO:10, 10A	12
(K)FYEAFSK(N) SEQ ID NO:11, 11A	12
(K)IDIIPNPQER(T) SEQ ID NO:12, 12A	12
(K)HFSVEGQLEFR(A) SEQ ID NO:13, 13A	12
(R)GVVDSEDLPLNISR(E) SEQ ID NO:14, 14A	12
(R)YHTSQSGDEMTSLSEYVSR(M) SEQ ID NO:15, 15A	12
(K)SIYYITGESKEQVANSAFVER(V) SEQ ID NO:16, 16A	12
(K)VTISNR(L) SEQ ID NO:17, 17A	12
(R)ALLFIPR(R) SEQ ID NO:18, 18A	12
(K)FYEAFSK(N) SEQ ID NO:19, 19A	12
(K)IDIIPNPQERT(T) SEQ ID NO:20, 20A	12
(K)HFSVEGQLEFR(A) SEQ ID NO:21, 21A	12
(R)GVVDSEDLPLNISR(E) SEQ ID NO:22, 22A	12
(R)YHTSQS-DEMTSLSEYVSR(M) SEQ ID NO:23, 23A	12
(K)SIYYITGESKEQVANSAFVER(V) SEQ ID NO:24, 24A	12
(K)VTISNR(L) SEQ ID NO:25, 25A	12
(R)ALLFVPR(R) SEQ ID NO:26, 26A	12
(K)FYEAFSK(N) SEQ ID NO:27, 27A	12
(K)IDILPNPQER(T) SEQ ID NO:28, 28A	12
(K)HESVEGQLEFR(A) SEQ ID NO:29, 29A	12
(R)GVVDSEDLPLNISR(E) SEQ ID NO:30, 30A	12
(K)SIYYITGESKEQVANSAFVER(V) SEQ ID NO:32, 32A	12
(K)VTISNR(L) SEQ ID NO:33, 33A	12
(R)ALLFIPR(R) SEQ ID NO:34, 34A	12
(K)FYEAFSK(N) SEQ ID NO:35, 35A	12
(K)HFSVEGQLEFR(A) SEQ ID NO:36, 36A	12
(R)GVVDSEDLPLNISR(E) SEQ ID NO:37, 37A	12
(R)YMTSQSGDEMTSLSEYVSR(M) SEQ ID NO:38, 38A	12
(K)SIYYITGESKEQVANSAFVER(V) SEQ ID NO:39, 39A	12

Peptide Sequence	Fig. No.
(K)IDILPNPQER(T) SEQ ID NO:40, 40A	12
(K)IDILPNPQER(T) SEQ ID NO:41, 41A	12
(K)IDIIPNPQER(T) SEQ ID NO:42, 42A	12
(K)IDILPNPQER(T) SEQ ID NO:43, 43A	12
(K)IDILPNPQER(T) SEQ ID NO:44, 44A	12
(K)IDIIPNPQER(T) SEQ ID NO:45, 45A	12
(R)ALLFVPR(R) SEQ ID NO:46, 46A	12
(R)ALLFYPR(R) SEQ ID NO:47, 47A	12
(K)AILFVPR(R) SEQ ID NO:48, 48A	12
(R)ALLFVPR(R) SEQ ID NO:49, 49A	12
(R)ALLFVPR(R) SEQ ID NO:50, 50A	12
(K)AILFVPR(R) SEQ ID NO:51, 51A	12
(K)VLTFYR(K) SEQ ID NO:52, 52A	13
(K)NTVQGFKR(F) SEQ ID NO:53, 53A	13
(K)VLATAFDTTLGGR(K) SEQ ID NO:54, 54A	13
(K)NAVEEYVYEMR(D) SEQ ID NO:55, 55A	13
(R)AGGIETIANEYSDR(C) SEQ ID NO:56, 56A	13
(R)EFSITDVVPYPISLR(W) SEQ ID NO:57, 57A	13
(R)WNSPAEEGSSDCEVFPK(N) SEQ ID NO:58, 58A	13
(K)VLTFYR(K) SEQ ID NO:59, 59A	13
(K)NTVQGFKR(F) SEQ ID NO:60, 60A	13
(K)QVYVDKLAELK(S) SEQ ID NO:61, 61A	13
(K)VLATAFDTTLGGR(K) SEQ ID NO:62, 62A	13
(K)NAVEEYVYEMR(D) SEQ ID NO:63, 63A	13
(R)AGGIETIANEYSDR(C) SEQ ID NO:64, 64A	13
(R)EFSITDVVPYPISLR(W) SEQ ID NO:65, 65A	13
(K)VLTFYR(K) SEQ ID NO:66, 66A	13
(K)NTVQGFKR(F) SEQ ID NO:67, 67A	13
(K)QVYVDKLAELK(S) SEQ ID NO:68, 68A	13
(K)VLATAFDTTIGGR(K) SEQ ID NO:69, 69A	13

Peptide Sequence	Fig. No.
(K)NAVEEYVYEMR(D) SEQ ID NO:70, 70A	13
(R)AGGIETIANEYSDR(C) SEQ ID NO:71, 71A	13
(R)EFSITDVVPYPISLR(W) SEQ ID NO:72, 72A	13
(K)VFHGLK(S) SEQ ID NO:74, 74A	14
(K)YAISMAR(K) SEQ ID NO:75, 75A	14
(R)VNKPPVPK(L) SEQ ID NO:76, 76A	14
(K)LSPEELLLR(W) SEQ ID NO:77, 77A	14
(K)IKVPVDWNR(V) SEQ ID NO:78, 78A	14
(R)QFVTATDVVR(G) SEQ ID NO:79, 79A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:80, 80A	14
(K)MINLSVPDTIDER(T) SEQ ID NO:81, 81A	14
(R)VYALPEDLVEVNPK(M) SEQ ID NO:82, 82A	14
(K)FSLVGIAGQDLNEGNR(T) SEQ ID NO:83, 83A	14
(K)GDEEGIPAVVIDMSGLR(E) SEQ ID NO:84, 84A	14
(K)VFHGLK(S) SEQ ID NO:85, 85A	14
(K)YAISMAR(K) SEQ ID NO:86, 86A	14
(R)VNKPPYPK(L) SEQ ID NO:87, 87A	14
(K)LSPEELLLR(W) SEQ ID NO:88, 88A	14
(K)IKVPVDWMR(V) SEQ ID NO:89, 89A	14
(R)QFVTATDVVR(G) SEQ ID NO:90, 90A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:91, 91A	14
(K)MINLSVPDTIDER(T) SEQ ID NO:93, 93A	14
(R)VYALPEDLVEVNPK(M) SEQ ID NO:94, 94A	14
(K)ESLVGIAGQDLNEGNR(T) SEQ ID NO:95, 95A	14

Peptide Sequence	Fig. No.
(K)GDEEGIPAVVIDMSGLR(E) SEQ ID NO:96, 96A	14
(K)VFHGLK(T) SEQ ID NO:97, 97A	14
(K)YAISMAR(K) SEQ ID NO:98, 98A	14
(R)VNKPPYPK(L) SEQ ID NO:99, 99A	14
(K)LSPEELLLR(W) SEQ ID NO:100, 100A	14
(K)IKVPVDWNR(V) SEQ ID NO:101, 101A	14
(R)QFVTATDVVR(G) SEQ ID NO:102, 102A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:103, 103A	14
(K)MINLSVPDTIDER(T) SEQ ID NO:104, 104A	14
(R)VYALPEDLVEVNPK(M) SEQ ID NO:105, 105A	14
(K)GDEEGIPAVVIDMSGLR(E) SEQ ID NO:106, 106A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:107, 107A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:108, 108A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:109, 109A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:110, 110A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:111, 111A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:112, 112A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:113, 113A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:114, 114A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:115, 115A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:116, 116A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:117, 117A	14
(R)NWMNSLGVNPR(V) SEQ ID NO:118, 118A	14

Fugetactic proteins can be in secreted or non-secreted form. That is, in the *in vitro* or *in vivo* methods provided herein, either the non-secreted or secreted forms of the proteins can be used. Secreted proteins can contain a signal or secretory sequence. These sequences are known in the art. Secreted proteins can be actively or passively secreted. Accordingly, one of ordinary skill in the art is able to construct a secreted protein by altering the sequence of a non-secreted form of the protein. For example, the endoplasmic reticulum retention signal of a non-secreted heat shock protein can be removed or altered to promote secretion of the HSP (see Strbo, et al., Heat shock fusion protein gp96-Ig mediates strong CD8 CTL expansion *in vivo*, Am. J. of Reproductive Immunol. 2002, 48: 220-225). The sequence of a non-secreted HSP can also be altered by providing a signal or secretory sequence. Unaltered proteins with retention signals, however, can also be used and do not necessarily have to be altered.

Methods of producing a substantially pure fugetactic agent, such as a fugetactic polypeptide are provided. The fugetactic agent, e.g. a fugetactic polypeptide, can be isolated from a non-homogenous proteinaceous solution such as a cell culture supernatant or cell homogenate. Tumor cells, such as thymoma cells, can be isolated from a subject by the disaggregation of a piece of tumor tissue, and forming cell suspensions. Alternatively, tumor cells may be tumor cell lines. Disaggregation of a tissue or a population of cells can be readily accomplished using techniques known to those skilled in the art. For example, the tissue or organ can be disaggregated mechanically and/or treated with digestive enzymes and/or chelating agents that weaken the connections between neighboring cells, making it possible to disperse the tissue into a suspension of individual cells without appreciable cell breakage. Enzymatic dissociation can be accomplished by mincing the tissue and treating the minced tissue with any of a number of digestive enzymes either alone or in combination. These include but are not limited to trypsin, chymotrypsin, collagenase, elastase, and/or hyaluronidase, DNase, pronase, dispase, etc. Mechanical disruption can also be accomplished by a number of methods including, but not limited to, the use of grinders, blenders, sieves, homogenizers, pressure cells, or insonators to name but a few. For a review of tissue disaggregation techniques, see Freshney, *Culture of Animal Cells*, A Manual of Basic Techniques, 2d Ed., A. R. Liss, Inc., New York, 1987, Ch. 9, pp. 107-126.

Once the tissue has been reduced to a suspension of individual cells, the suspension optionally can be fractionated into subpopulations from which the desired cell and/or elements can be obtained. This also may be accomplished using standard techniques for cell separation including, but not limited to, cloning and selection of specific cell types, selective destruction of unwanted cells (negative selection), separation based upon differential cell agglutinability in the mixed population, freeze-thaw procedures, differential adherence properties of the cells in the mixed population, filtration, conventional and zonal centrifugation, centrifugal elutriation (counter-streaming centrifugation), unit gravity separation, countercurrent distribution, electrophoresis and fluorescence-activated cell sorting. For a review of clonal selection and cell separation techniques, see Freshney, *Culture of Animal Cells*, A Manual of Basic Techniques, 2d Ed., A. R. Liss, Inc., New York, 1987, Ch. 11 and 12, pp. 137-168.

The cells can also be cultured in order to prepare a culture supernatant that contains the fugetactic agent. Cells can be cultured in an appropriate nutrient medium under conditions that are metabolically favorable for the growth of the cells. As used herein, the phrase 'metabolically favorable conditions" refers to conditions that maintain cell viability and promote secretion of factors into the culture media. Such conditions include growth in nutrient medium at 37°C in a 5% CO_2 incubator with greater than 90% humidity. Many commercially available media, such as RPMI 1640, Fisher's, Iscove's, McCoy's, Dulbecco's Modified Eagle's Medium, etc., and the like, which may or may not be supplemented with serum, may be suitable for use as nutrient medium. Antibiotics such as penicillin and streptomycin may also be included. In preferred embodiments, the tumor cells are cultured first, for a brief period of time in the presence of 10% serum. The medium is then changed to a serum-free medium in order to minimize the number of extraneous (e.g. non-tumor cell derived) agents that would be present in the medium under continuous culture. The serum-free cell medium becomes a cell conditioned medium (supernatant) upon the growth of the cells. In general, these cell suspensions can be cultured according to standard cell culture techniques. In small scale, the cultures can be contained in culture plates, flasks, and dishes. In larger scale, the cultures can be contained in roller bottles, spinner flasks and other large scale culture vessels such as fermenters. Culturing in a three-dimensional, porous,

solid matrix may also be used. Preferably, the cells are grown to confluency (for example, at a concentration of 10^6 cells/mL medium in suspension).

The supernatant from the cell culture is then isolated. The supernatant may be harvested by aspiration, or by centrifugation of the cell culture to remove cells. The cultures can also be filtered to remove cells and cell debris. The supernatant is then fractionated and each fraction is contacted with a cell with migratory capacity. A "cell with migratory capacity" or a "migratory cell" is a cell that is capable of either moving towards or away from a stimulus. They can respond to a variety of chemotactic signals, including chemoattractive and chemorepulsive (fugetactic) signals.

A "cell with migratory capacity" is an eukaryotic cell that includes, but is not limited to, a cell of hematopoietic origin, a cell of neural origin, a cell of epithelial origin, a cell involved in angiogenesis, a cell of mesenchymal origin, an embryonic stem cell, or a germ cell. Such cells with migratory capacity can respond to a variety of chemotactic signals, including chemoattractive and chemorepulsive (fugetactic) signals. The response typically involves changes in the actin cytoskeleton.

Cells of "hematopoietic origin" include, but are not limited to, pluripotent stem cells, multipotent progenitor cells and/or progenitor cells committed to specific hematopoietic lineages. The progenitor cells committed to specific hematopoietic lineages may be of T cell lineage, B cell lineage, dendritic cell lineage, Langerhans cell lineage and/or lymphoid tissue-specific macrophage cell lineage. The hematopoietic cells may be derived from a tissue such as bone marrow, peripheral blood (including mobilized peripheral blood), umbilical cord blood, placental blood, fetal liver, embryonic cells (including embryonic stem cells), aortal-gonadal-mesonephros derived cells, and lymphoid soft tissue. Lymphoid soft tissue includes the thymus, spleen, liver, lymph node, skin, tonsil and Peyer's patches. In other embodiments, the "hematopoietic origin" cells may be derived from *in vitro* cultures of any of the foregoing cells, and in particular *in vitro* cultures of progenitor cells.

"Immune cells" as used herein are cells of hematopoietic origin that are involved in the specific recognition of antigens. Immune cells include antigen presenting cells (APCs), such as dendritic cells or macrophages, B cells, T cells, etc. "T cells" as used herein include T cells of a $CD4^{lo}CD8^{hi}CD69^{+}TCR^{+}$,

$CD4^{hi}CD8^{lo}CD69^{+}TCR^{+}$, $CD4^{+}CD3^{+}$ RO $^{+}$ and/or $CD8^{+}CD3^{+}$ RO $^{+}$ phenotype. T cells include cytotoxic T lymphocytes (CTLs).

Cells of neural origin, include neurons and glia, and/or cells of both central and peripheral nervous tissue that express RR/B (see, U.S. Patent 5,863,744, entitled: "Neural cell protein marker RR/B and DNA encoding same," issued January 26, 1999, to Avraham, et al.).

Cells of epithelial origin, include cells of a tissue that covers and lines the free surfaces of the body. Such epithelial tissue includes cells of the skin and sensory organs, as well as the specialized cells lining the blood vessels, gastrointestinal tract, air passages, ducts of the kidneys and endocrine organs.

Cells of mesenchymal origin include cells that express typical fibroblast markers such as collagen, vimentin and fibronectin.

Cells involved in angiogenesis are cells that are involved in blood vessel formation and include cells of epithelial origin and cells of mesenchymal origin.

An embryonic stem cell is a cell that can give rise to cells of all lineages; it also has the capacity to self-renew.

A germ cell is a cell specialized to produce haploid gametes. It is a cell further differentiated than a stem cell, that can still give rise to more differentiated germ-line cells.

The tumor cell may be of a cancer or tumor type demonstrated to escape immune recognition. Such cancers or tumors include cancer of the thymus, as well as biliary tract cancer; brain cancer, including glioblastomas and medulloblastomas; breast cancer; cervical cancer; choriocarcinoma; colon cancer; colorectal cancer; endometrial cancer; esophageal cancer; gastric cancer; glioma; hematological neoplasms, including acute lymphocytic, lymphoid cell-derived leukemia and myelogenous leukemia; multiple myeloma; AIDS associated leukemias and adult T cell leukemia and lymphoma; intraepithelial neoplasms, including Bowen's disease and Paget's disease; liver cancer (hepatocarcinoma); lung cancer (e.g. Lewis lung carcinoma); lymphomas, including Hodgkin's disease and lymphocytic lymphomas; neuroblastomas; oral cancer, including squamous cell carcinoma; ovarian cancer, including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells; pancreatic cancer; prostate cancer; rectal cancer; sarcomas, including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma and

osteosarcoma; skin cancer, including melanoma, Kaposi's sarcoma, basocellular cancer and squamous cell cancer; testicular cancer, including germinal tumors (seminoma and non-seminoma (e.g., teratomas or choriocarcinomas)), stromal tumors and germ cell tumors; thyroid cancer, including thyroid adenocarcinoma and medullar carcinoma; and renal cancer including adenocarcinoma and Wilms tumor.

The tumor cell isolate can be isolated from the supernatants of the above-described cell cultures. The entire culture may be homogenized and subjected to the steps described herein for isolation of a fugetactic agent, such as a fugetactic polypeptide. In still other embodiments, the fugetactic agent takes the form of a diluted supernatant from these tumor cells. The supernatant can be diluted about 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 15-, 20-, 25-, 30-, 40-, 50-, 60-, 70-, 80-, 90-, 100-, 125-, 150- or 200-fold, etc.

The fugetactic agent containing tumor cell supernatant can be fractionated according to standard chromatographic procedures to facilitate isolation of the fugetactic agent. One of ordinary skill in the art will be familiar with such procedures that include, but are not limited to, size-exclusion chromatography, FPLC, HPLC, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, immune-affinity chromatography, etc.

The fugetactic response of the T cells to the fugetactic agent can be measured as described herein, or according to the transmigration assays described in greater detail in the Examples. Other suitable methods will be known to one of ordinary skill in the art and can be employed using only routine experimentation.

The fractions which are positive for fugetactic activity can be subjected to additional rounds of screening using the foregoing methodology. The purity of the fraction can be assessed after each round of culture stimulation by subjecting an aliquot of the fraction to SDS-PAGE or other analytical method for visualizing the mixture of constituents in the fraction. The nature of the fugetactic agent (e.g., protein, nucleic acid, lipid, carbohydrate etc.), can be confirmed at any time by treating an aliquot of a positive fraction with non-specific degradative enzymes for the foregoing classes of molecules and testing the treated fraction in the same assays detailed above.

The fugetactic agent can then be further isolated if desired using immunological and molecular biological methods (*see, e.g. Molecular Cloning: A*

Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York). For example, a fraction positive for the tumor cell isolate which is sufficiently purified can be subjected to protein sequencing according to standard methods. For example, the fraction can be subjected to SDS-PAGE, transferred to a membrane such as polyvinylidene fluoride by electroblotting, and N-terminal amino sequence determined by Edman degradation. Any sequence information can be used to screen databases for homology to existing proteins and also to generate degenerate nucleic acids useful for screening a cDNA library by standard methods such as colony hybridization or polymerase chain reaction.

Alternatively, the positive fraction can be used to generate antibodies specific for the fugetactic agent. Such antibodies can then be used in expression cloning protocols, Western blots, and other techniques useful in isolation of the fugetactic agent. In the foregoing methods, any cDNA libraries, expression libraries, etc., are created from tumor cells, preferably thymoma cells, and even more preferably EL4 thymoma cells.

The invention also provides agents that selectively bind to and preferably inhibit the activity of the fugetactic agents provided herein (i.e., "anti-fugetactic agents"). Anti-fugetactic agents can be isolated peptides, antibodies or antigen-binding fragments thereof or other inhibitors, e.g. small molecules. These agents include HSP inhibitors, such as radicicol, Geldanamycin, 17-A-GA, herbimycin A, PU3, novobiocin and G-coupled pertussis toxin. Other HSP inhibitors are known in the art. In some embodiments the anti-fugetactic agent is not a benzoquinoid ansamycin. In still other embodiments the anti-fugetactic agent is not radicicol. In yet other embodiments the anti-fugetactic agent is not geldanamycin or 17-A-GA. In still other embodiments the anti-fugetactic agent is not herbimycin A. In yet other embodiments the anti-fugetactic agent is not PU3. In still other instances the anti-fugetactic agent is not novobiocin. In other instances the anti-fugetactic agent is not pertussis toxin. Anti-fugetactic agents can also be molecules that competitively or non-competitively bind to the receptors of the fugetactic proteins provided herein. For instance receptors have been postulated for HSPs, which are, for example, CD91, CD36 and tlr4.

The anti-fugetactic agents of the invention can be discovered with routine screening methods. The invention therefore provides a method of screening for an anti-fugetactic agent. Screening for an anti-fugetactic agent includes combining a migratory cell, such as an immune cell, with a fugetactic agent and determining a control level of fugetactic activity. Combining the fugetactic agent, migratory cell and a candidate anti-fugetactic agent provides a test level of activity, which can then be compared with the control level to determine whether or not the candidate anti-fugetactic agent exhibits the desired activity.

Further, it is possible to isolate proteins which bind to the fugetactic agents disclosed herein, including antibodies and other binding partners of the fugetactic agents such as receptors. The proteins which bind to the fugetactic agents can be used, for example, in screening assays to detect the presence or absence of fugetactic agents as well as in purification protocols to isolate fugetactic agents. The binding proteins also can be used to block the effects of the fugetactic agents. Such assays can be used to confirm the specificity of binding.

The invention, therefore, embraces binding agents which, for example, can be antibodies or fragments of antibodies having the ability to selectively bind to fugetactic agents. These antibodies can also inhibit the fugetactic properties of the agents described above. In important embodiments, the binding agents bind selectively to a fugetactic agent. In further important embodiments, the binding agents bind selectively to a HSP, HSPLP, L-plastin or LPLP. These binding agents can inhibit repulsion of immune cells. Antibodies include polyclonal and monoclonal antibodies, prepared according to conventional methodology.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an $F(ab')_2$ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has

been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. Thus, for example, PCT International Publication Number WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for $F(ab')_2$, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric $F(ab')_2$ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-

human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

Thus, the invention provides other peptides or polypeptides of numerous size and type that bind specifically to the fugetactic agents provided herein or complexes of fugetactic agents and their binding partners. These polypeptides may be derived also from sources other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

As detailed herein, the foregoing antibodies and other binding molecules may be used for example to identify tissues expressing protein or to purify protein. Antibodies also may be coupled to specific diagnostic labeling agents for imaging of cells and tissues that express a fugetactic agent as provided herein.

Anti-fugetactic agents also include molecules which reduce the expression level and/or function level of the fugetactic agents of the invention. Such molecules include antisense molecules and RNAi molecules. The use of RNA interference or "RNAi" involves the use of double-stranded RNA (dsRNA) to block gene expression. (see: Sui, G, et al, Proc Natl. Acad. Sci U.S.A. 99:5515-5520,2002). Methods of applying antisense and RNAi strategies in embodiments of the invention would be understood by one of ordinary skill in the art.

The fugetactic agents of the invention are useful for modulating cell migration away from specific sites in a subject. Thus, in one aspect, the invention involves a method of promoting fugetaxis at a specific site by administrating a fugetactic agent provided herein in an effective amount to promote fugetaxis at the site.

As used herein, a subject is a human subject, a non-human primate, a horse, a cow, a pig, a sheep, a bird such as a chicken, a dog, a cat, a fish, etc. In preferred embodiments, the subject is a human.

A "site" can be any place in a subject where the promotion or inhibition of fugetaxis is needed. For example, such sites include a site of inflammation, infection, an autoimmune reaction, an allergic reaction, transplantation (e.g. a transplanted organ or tissue), implant (e.g. a medical or prosthetic device or a stent) and a tumor. One of ordinary skill in the art would be able to easily determine at which site the promotion or inhibition of fugetaxis would be beneficial. These sites include, but are not limited to, a site of inflammation, a site of an autoimmune reaction and a site of a transplanted organ or tissue. "Inflammation" as used herein, is a localised protective response elicited by a foreign (non-self) antigen, and/or by an injury or destruction of tissue(s), which serves to destroy, dilute or sequester the foreign antigen, the injurious agent, and/or the injured tissue. Inflammation occurs when tissues are injured by viruses, bacteria, trauma, chemicals, heat, cold, or any other harmful stimuli. In such instances, the classic weapons of the immune system (T cells, B cells, macrophages) interface with cells and soluble products that are mediators of inflammatory responses (neutrophils, eosinophils, basophils, kinin and coagulation systems, and complement cascade).

A typical inflammatory response is characterized by (i) migration of leukocytes at the site of antigen (injury) localization; (ii) specific and nonspecific recognition of "foreign" and other (necrotic/injured tissue) antigens mediated by B and T lymphocytes, macrophages and the alternative complement pathway; (iii) amplification of the inflammatory response with the recruitment of specific and nonspecific effector cells by complement components, lymphokines and monokines, kinins, arachidonic acid metabolites, and mast cell/basophil products; and (iv) macrophage, neutrophil and lymphocyte participation in antigen destruction with ultimate removal of antigen particles (injured tissue) by phagocytosis. The ability of the immune system to discriminate between "self" and "non-self"(foreign) antigens is therefore vital to the functioning of the immune system as a specific defense against "non-self" antigens.

"Non-self" antigens are those antigens on substances entering a subject, or exist in a subject but are detectably different or foreign from the subject's own

constituents, whereas "self" antigens are those which, in the healthy subject, are not detectably different or foreign from its own constituents. However, under certain conditions, including in certain disease states, an individual's immune system will identify its own constituents as "non-self," and initiate an immune response against "self-antigens," at times causing more damage or discomfort as from, for example, an invading microbe or foreign material, and often producing serious illness in a subject.

In another important embodiment, the inflammation is caused by an immune response against "self-antigen," and the subject in need of treatment according to the invention has an autoimmune disease. "Autoimmune disease" as used herein, results when a subject's immune system attacks its own organs or tissues, producing a clinical condition associated with the destruction of that tissue, as exemplified by diseases such as rheumatoid arthritis, uveitis, insulin-dependent diabetes mellitus, hemolytic anemias, rheumatic fever, Crohn's disease, Guillain-Barre syndrome, psoriasis, thyroiditis, Graves' disease, myasthenia gravis, glomerulonephritis, autoimmune hepatitis, multiple sclerosis, systemic lupus erythematosus, etc.

Autoimmune disease may be caused by a genetic predisposition alone, by certain exogenous agents (e.g., viruses, bacteria, chemical agents, etc.), or both. Some forms of autoimmunity arise as the result of trauma to an area usually not exposed to lymphocytes, such as neural tissue or the lens of the eye. When the tissues in these areas become exposed to lymphocytes, their surface proteins can act as antigens and trigger the production of antibodies and cellular immune responses which then begin to destroy those tissues. Other autoimmune diseases develop after exposure of a subject to antigens which are antigenicly similar to, that is cross-reactive with, the subject's own tissue. In rheumatic fever, for example, an antigen of the streptococcal bacterium, which causes rheumatic fever, is cross-reactive with parts of the human heart. The antibodies cannot differentiate between the bacterial antigens and the heart muscle antigens, consequently cells with either of those antigens can be destroyed.

Other autoimmune diseases, for example, insulin-dependent diabetes mellitus (involving the destruction of the insulin producing beta cells of the islets of Langerhans), multiple sclerosis (involving the destruction of the conducting fibers of the nervous system) and rheumatoid arthritis (involving the destruction of the joint-

lining tissue), are characterized as being the result of a mostly cell-mediated autoimmune response and appear to be due primarily to the action of T cells (See, Sinha et al., *Science,* 1990, 248:1380). Yet others, such as myesthenia gravis and systemic lupus erythematosus, are characterized as being the result of primarily a humoral autoimmune response. Nevertheless, inhibition of migration of immune cells to a specific site of inflammation involved in any of the foregoing conditions according to the invention, is beneficial to the subject since it inhibits escalation of the inflammatory response, protecting the specific site (e.g., tissue) involved, from "self-damage."

In a further important embodiments, the inflammation is caused by an immune response against "non-self-antigens" (including antigens of necrotic self-material), and the subject in need of treatment according to the invention is a transplant recipient, has atherosclerosis, has suffered a myocardial infarction and/or an ischemic stroke, has an abscess, and/or has myocarditis. This is because after cell (or organ) transplantation, or after myocardial infarction or ischemic stroke, certain antigens from the transplanted cells (organs), or necrotic cells from the heart or the brain, can stimulate the production of immune lymphocytes and/or autoantibodies, which later participate in inflammation/rejection (in the case of a transplant), or attack cardiac or brain target cells causing inflammation and aggravating the condition (Johnson et al., *Sem. Nuc. Med.* 1989, 19:238; Leinonen et al., *Microbiol. Path.*,1990, 9:67; Montalban et al., *Stroke*, 1991, 22:750).

As used herein a "site of an allergic reaction" is any location, local or systemic, where there is an allergic response to an allergen. Allergic reactions in man and animals has been extensively studied and the basic immune mechanisms involved are well known. Allergic conditions or diseases in humans include but are not limited to eczema, allergic rhinitis or coryza, hay fever, conjunctivitis, bronchial or allergic asthma, urticaria (hives) and food allergies; atopic dermatitis; anaphylaxis; drug allergy; angioedema; and allergic conjunctivitis. An allergic reaction may be local or systemic anaphylaxis.

The generic name for molecules that cause an allergic reaction is allergen. There are numerous species of allergens. The allergic reaction occurs when tissue-sensitizing immunoglobulin of the IgE type reacts with foreign allergen. The IgE antibody is bound to mast cells and/or basophils, and these specialized cells release

chemical mediators (vasoactive amines) of the allergic reaction when stimulated to do so by allergens bridging the ends of the antibody molecule. Histamine, platelet activating factor, arachidonic acid metabolites, and serotonin are among the best known mediators of allergic reactions in man. Histamine and the other vasoactive amines are normally stored in mast cells and basophil leukocytes. The mast cells are dispersed throughout animal tissue and the basophils circulate within the vascular system. These cells manufacture and store histamine within the cell unless the specialized sequence of events involving IgE binding occurs to trigger its release.

A method of repelling immune cells from a material surface is also provided. "Material surfaces" as used herein, include, but are not limited to, medical devices, dental and orthopedic prosthetic implants, artificial valves, and organic implantable tissue such as a stent, allogeneic and/or xenogeneic tissue, organ and/or vasculature. Material surfaces also encompass tissues that are produced through tissue engineering technology.

Implantable prosthetic devices have been used in the surgical repair or replacement of internal tissue for many years. Orthopedic implants include a wide variety of devices, each suited to fulfill particular medical needs. Examples of such devices are hip joint replacement devices, knee joint replacement devices, shoulder joint replacement devices, and pins, braces and plates used to set fractured bones. Some contemporary orthopedic and dental implants, use high performance metals such as cobalt-chrome and titanium alloy to achieve high strength. These materials are readily fabricated into the complex shapes typical of these devices using mature metal working techniques including casting and machining.

The material surface is coated with an amount of a fugetactic agent of the invention effective to repel immune cells. In important embodiments, the material surface is part of an implant. In important embodiments, in addition to a fugetactic agent, the material surface may also be coated with a cell-growth potentiating agent, an anti-infective agent, and/or an anti-inflammatory agent.

According to another aspect of the invention, a method of treating infertility and premature labor, including premature delivery and impending miscarriage, is provided. The method involves administering to a subject in need of such treatment a fugetactic agent in an amount effective to inhibit immune cells from migrating close to a germ cell (including an egg, a sperm, a fertilized egg, or an implanted

embryo) in the subject. In further embodiments, the administration is local to a germ cell-containing site of the subject.

According to a further aspect of the invention, a method of contraception in a subject, is provided. The method involves administering to a subject in need of such treatment, an anti-fugetactic agent in an amount effective to inhibit migration of germ cells in the subject. In further embodiments, the administration is local to a germ cell-containing site of the subject.

According to another aspect of the invention, a method of inhibiting tumor cell metastasis in a subject is provided. The method involves locally administering to a tumor site in a subject in need of such treatment an anti-fugetactic agent in an amount effective to inhibit metastasis of tumor cells from the tumor site in the subject.

According to another aspect of the invention, a method of inhibiting endothelial cell migration to a tumor site in a subject, is provided. The method involves locally administering to an area surrounding a tumor site in a subject in need of such treatment a fugetactic agent in an amount effective to inhibit endothelial cell migration to the tumor site in the subject. In certain embodiments, the area surrounding the tumor site is not immediate to the tumor site. Important fugetactic agents are as described herein.

The methods of the invention also include a method of eliciting or enhancing a local immune response by administering an anti-fugetactic agent of the invention in an amount effective to inhibit immune cell specific fugetactic activity at a specific site in a subject thereby enhancing a local immune response. In some embodiments, the specific site is a site of an infection. Efficient recruitment of immune cells to help eliminate the infection is beneficial.

In further embodiments, the specific site is a tumor. It is beneficial to enhance the migration of immune cells to the tumor site, as well as to maintain such cells at this site. A tumor site can be any site where cancer cells are present in a subject. In further embodiments, co-administration of anti-cancer agents other than anti-fugetactic agents is also provided.

In another embodiment the specific site is a germ cell site where the recruitment of immune cells to the specific sites will help eliminate unwanted germ cells, and/or implanted and non-implanted embryos. In further embodiments, co-

administration of contraceptive agents other than anti-fugetactic agents is also provided. Non-anti-fugetactic contraceptive agents are well known in the art.

The foregoing methods of therapy may include co-administration of a non-fugetactic therapeutic agent together with a fugetactic agent or anti-fugetactic agent of the invention that can act cooperatively, additively, or synergistically with the fugetactic agent or anti-fugetactic agent of the invention to promote or inhibit the migration of cells from a specific site in a subject. According to some embodiments, a fugetactic agent or anti-fugetactic agent is administered substantially simultaneously with a non-fugetactic therapeutic agent. By "substantially simultaneously," it is meant that the fugetactic agent is administered to the subject close enough in time with the administration of the non-fugetactic therapeutic agent, whereby the non-fugetactic therapeutic agent may exert a potentiating effect on migration activity of the fugetactic agent or anti-fugetactic agent. Thus, by substantially simultaneously it is meant that the fugetactic agent or anti-fugetactic agent is administered before, at the same time, and/or after the administration of the non-fugetactic therapeutic agent. In some embodiments the fugetactic agents or anti-fugetactic agents can be administered as a polypeptide or as a nucleic acid, which expresses the fugetactic agent or anti-fugetactic agent.

An anti-infectious agent as used herein is an agent which reduces the activity of or kills a microorganism and includes: Aztreonam; Chlorhexidine Gluconate; Imidurea; Lycetamine; Nibroxane; Pirazmonam Sodium; Propionic Acid; Pyrithione Sodium; Sanguinarium Chloride; Tigemonam Dicholine; Acedapsone; Acetosulfone Sodium; Alamecin; Alexidine; Amdinocillin; Amdinocillin Pivoxil; Amicycline; Amifloxacin; Amifloxacin Mesylate; Amikacin; Amikacin Sulfate; Aminosalicylic acid; Aminosalicylate sodium; Amoxicillin; Amphomycin; Ampicillin; Ampicillin Sodium; Apalcillin Sodium; Apramycin; Aspartocin; Astromicin Sulfate; Avilamycin; Avoparcin; Azithromycin; Azlocillin; Azlocillin Sodium; Bacampicillin Hydrochloride; Bacitracin; Bacitracin Methylene Disalicylate; Bacitracin Zinc; Bambermycins; Benzoylpas Calcium; Berythromycin; Betamicin Sulfate; Biapenem; Biniramycin; Biphenamine Hydrochloride; Bispyrithione Magsulfex; Butikacin; Butirosin Sulfate; Capreomycin Sulfate; Carbadox; Carbenicillin Disodium; Carbenicillin Indanyl Sodium; Carbenicillin Phenyl Sodium; Carbenicillin Potassium; Carumonam Sodium; Cefaclor; Cefadroxil;

Cefamandole; Cefamandole Nafate; Cefamandole Sodium; Cefaparole; Cefatrizine; Cefazaflur Sodium; Cefazolin; Cefazolin Sodium; Cefbuperazone; Cefdinir; Cefepime; Cefepime Hydrochloride; Cefetecol; Cefixime; Cefmenoxime Hydrochloride; Cefmetazole; Cefmetazole Sodium; Cefonicid Monosodium; Cefonicid Sodium; Cefoperazone Sodium; Ceforanide; Cefotaxime Sodium; Cefotetan; Cefotetan Disodium; Cefotiam Hydrochloride; Cefoxitin; Cefoxitin Sodium; Cefpimizole; Cefpimizole Sodium; Cefpiramide; Cefpiramide Sodium; Cefpirome Sulfate; Cefpodoxime Proxetil; Cefprozil; Cefroxadine; Cefsulodin Sodium; Ceftazidime; Ceftibuten; Ceftizoxime Sodium; Ceftriaxone Sodium; Cefuroxime; Cefuroxime Axetil; Cefuroxime Pivoxetil; Cefuroxime Sodium; Cephacetrile Sodium; Cephalexin; Cephalexin Hydrochloride; Cephaloglycin; Cephaloridine; Cephalothin Sodium; Cephapirin Sodium; Cephradine; Cetocycline Hydrochloride; Cetophenicol; Chloramphenicol; Chloramphenicol Palmitate; Chloramphenicol Pantothenate Complex; Chloramphenicol Sodium Succinate; Chlorhexidine Phosphanilate; Chloroxylenol; Chlortetracycline Bisulfate; Chlortetracycline Hydrochloride; Cinoxacin; Ciprofloxacin; Ciprofloxacin Hydrochloride; Cirolemycin; Clarithromycin; Clinafloxacin Hydrochloride; Clindamycin; Clindamycin Hydrochloride; Clindamycin Palmitate Hydrochloride; Clindamycin Phosphate; Clofazimine; Cloxacillin Benzathine; Cloxacillin Sodium; Cloxyquin; Colistimethate Sodium; Colistin Sulfate; Coumermycin; Coumermycin Sodium; Cyclacillin; Cycloserine; Dalfopristin; Dapsone; Daptomycin; Demeclocycline; Demeclocycline Hydrochloride; Demecycline; Denofungin; Diaveridine; Dicloxacillin; Dicloxacillin Sodium; Dihydrostreptomycin Sulfate; Dipyrithione; Dirithromycin; Doxycycline; Doxycycline Calcium; Doxycycline Fosfatex; Doxycycline Hyclate; Droxacin Sodium; Enoxacin; Epicillin; Epitetracycline Hydrochloride; Erythromycin; Erythromycin Acistrate; Erythromycin Estolate; Erythromycin Ethylsuccinate; Erythromycin Gluceptate; Erythromycin Lactobionate; Erythromycin Propionate; Erythromycin Stearate; Ethambutol Hydrochloride; Ethionamide; Fleroxacin; Floxacillin; Fludalanine; Flumequine; Fosfomycin; Fosfomycin Tromethamine; Fumoxicillin; Furazolium Chloride; Furazolium Tartrate; Fusidate Sodium; Fusidic Acid; Gentamicin Sulfate; Gloximonam; Gramicidin; Haloprogin; Hetacillin; Hetacillin Potassium; Hexedine; Ibafloxacin; Imipenem; Isoconazole; Isepamicin; Isoniazid; Josamycin; Kanamycin

Sulfate; Kitasamycin; Levofuraltadone; Levopropylcillin Potassium; Lexithromycin; Lincomycin; Lincomycin Hydrochloride; Lomefloxacin; Lomefloxacin Hydrochloride; Lomefloxacin Mesylate; Loracarbef; Mafenide; Meclocycline; Meclocycline Sulfosalicylate; Megalomicin Potassium Phosphate; Mequidox; Meropenem; Methacycline; Methacycline Hydrochloride; Methenamine; Methenamine Hippurate; Methenamine Mandelate; Methicillin Sodium; Metioprim; Metronidazole Hydrochloride; Metronidazole Phosphate; Mezlocillin; Mezlocillin Sodium; Minocycline; Minocycline Hydrochloride; Mirincamycin Hydrochloride; Monensin; Monensin Sodium; Nafcillin Sodium; Nalidixate Sodium; Nalidixic Acid; Natamycin; Nebramycin; Neomycin Palmitate; Neomycin Sulfate; Neomycin Undecylenate; Netilmicin Sulfate; Neutramycin; Nifuradene; Nifuraldezone; Nifuratel; Nifuratrone; Nifurdazil; Nifurimide; Nifurpirinol; Nifurquinazol; Nifurthiazole; Nitrocycline; Nitrofurantoin; Nitromide; Norfloxacin; Novobiocin Sodium; Ofloxacin; Ormetoprim; Oxacillin Sodium; Oximonam; Oximonam Sodium; Oxolinic Acid; Oxytetracycline; Oxytetracycline Calcium; Oxytetracycline Hydrochloride; Paldimycin; Parachlorophenol; Paulomycin; Pefloxacin; Pefloxacin Mesylate; Penamecillin; Penicillin G Benzathine; Penicillin G Potassium; Penicillin G Procaine; Penicillin G Sodium; Penicillin V; Penicillin V Benzathine; Penicillin V Hydrabamine; Penicillin V Potassium; Pentizidone Sodium; Phenyl Aminosalicylate; Piperacillin Sodium; Pirbenicillin Sodium; Piridicillin Sodium; Pirlimycin Hydrochloride; Pivampicillin Hydrochloride; Pivampicillin Pamoate; Pivampicillin Probenate; Polymyxin B Sulfate; Porfiromycin; Propikacin; Pyrazinamide; Pyrithione Zinc; Quindecamine Acetate; Quinupristin; Racephenicol; Ramoplanin; Ranimycin; Relomycin; Repromicin; Rifabutin; Rifametane; Rifamexil; Rifamide; Rifampin; Rifapentine; Rifaximin; Rolitetracycline; Rolitetracycline Nitrate; Rosaramicin; Rosaramicin Butyrate; Rosaramicin Propionate; Rosaramicin Sodium Phosphate; Rosaramicin Stearate; Rosoxacin; Roxarsone; Roxithromycin; Sancycline; Sanfetrinem Sodium; Sarmoxicillin; Sarpicillin; Scopafungin; Sisomicin; Sisomicin Sulfate; Sparfloxacin; Spectinomycin Hydrochloride; Spiramycin; Stallimycin Hydrochloride; Steffimycin; Streptomycin Sulfate; Streptonicozid; Sulfabenz; Sulfabenzamide; Sulfacetamide; Sulfacetamide Sodium; Sulfacytine; Sulfadiazine; Sulfadiazine Sodium; Sulfadoxine; Sulfalene; Sulfamerazine; Sulfameter; Sulfamethazine; Sulfamethizole;

Sulfamethoxazole; Sulfamonomethoxine; Sulfamoxole; Sulfanilate Zinc; Sulfanitran; Sulfasalazine; Sulfasomizole; Sulfathiazole; Sulfazamet; Sulfisoxazole; Sulfisoxazole Acetyl; Sulfisoxazole Diolamine; Sulfomyxin; Sulopenem; Sultamicillin; Suncillin Sodium; Talampicillin Hydrochloride; Teicoplanin; Temafloxacin Hydrochloride; Temocillin; Tetracycline; Tetracycline Hydrochloride; Tetracycline Phosphate Complex; Tetroxoprim; Thiamphenicol; Thiphencillin Potassium; Ticarcillin Cresyl Sodium; Ticarcillin Disodium; Ticarcillin Monosodium; Ticlatone; Tiodonium Chloride; Tobramycin; Tobramycin Sulfate; Tosufloxacin; Trimethoprim; Trimethoprim Sulfate; Trisulfapyrimidines; Troleandomycin; Trospectomycin Sulfate; Tyrothricin; Vancomycin; Vancomycin Hydrochloride; Virginiamycin; Zorbamycin; Difloxacin Hydrochloride; Lauryl Isoquinolinium Bromide; Moxalactam Disodium; Ornidazole; Pentisomicin; and Sarafloxacin Hydrochloride.

In other embodiments, the non-fugetactic therapeutic agents are anti-inflammatory agents. Such anti-inflammatory agents include: Alclofenac; Alclometasone Dipropionate; Algestone Acetonide; Alpha Amylase; Amcinafal; Amcinafide; Amfenac Sodium; Amiprilose Hydrochloride; Anakinra; Anirolac; Anitrazafen; Apazone; Balsalazide Disodium; Bendazac; Benoxaprofen; Benzydamine Hydrochloride; Bromelains; Broperamole; Budesonide; Carprofen; Cicloprofen; Cintazone; Cliprofen; Clobetasol Propionate; Clobetasone Butyrate; Clopirac; Cloticasone Propionate; Cormethasone Acetate; Cortodoxone; Deflazacort; Desonide; Desoximetasone; Dexamethasone Dipropionate; Diclofenac Potassium; Diclofenac Sodium; Diflorasone Diacetate; Diflumidone Sodium; Diflunisal; Difluprednate; Diftalone; Dimethyl Sulfoxide; Drocinonide; Endrysone; Enlimomab; Enolicam Sodium; Epirizole; Etodolac; Etofenamate; Felbinac; Fenamole; Fenbufen; Fenclofenac; Fenclorac; Fendosal; Fenpipalone; Fentiazac; Flazalone; Fluazacort; Flufenamic Acid; Flumizole; Flunisolide Acetate; Flunixin; Flunixin Meglumine; Fluocortin Butyl; Fluorometholone Acetate; Fluquazone; Flurbiprofen; Fluretofen; Fluticasone Propionate; Furaprofen; Furobufen; Halcinonide; Halobetasol Propionate; Halopredone Acetate; Ibufenac; Ibuprofen; Ibuprofen Aluminum; Ibuprofen Piconol; Ilonidap; Indomethacin; Indomethacin Sodium; Indoprofen; Indoxole; Intrazole; Isoflupredone Acetate; Isoxepac; Isoxicam; Ketoprofen; Lofemizole Hydrochloride; Lornoxicam; Loteprednol

Etabonate; Meclofenamate Sodium; Meclofenamic Acid; Meclorisone Dibutyrate; Mefenamic Acid; Mesalamine; Meseclazone; Methylprednisolone Suleptanate; Morniflumate; Nabumetone; Naproxen; Naproxen Sodium; Naproxol; Nimazone; Olsalazine Sodium; Orgotein; Orpanoxin; Oxaprozin; Oxyphenbutazone; Paranyline Hydrochloride; Pentosan Polysulfate Sodium; Phenbutazone Sodium Glycerate; Pirfenidone; Piroxicam; Piroxicam Cinnamate; Piroxicam Olamine; Pirprofen; Prednazate; Prifelone; Prodolic Acid; Proquazone; Proxazole; Proxazole Citrate; Rimexolone; Romazarit; Salcolex; Salnacedin; Salsalate; Sanguinarium Chloride; Seclazone; Sermetacin; Sudoxicam; Sulindac; Suprofen; Talmetacin; Talniflumate; Talosalate; Tebufelone; Tenidap; Tenidap Sodium; Tenoxicam; Tesicam; Tesimide; Tetrydamine; Tiopinac; Tixocortol Pivalate; Tolmetin; Tolmetin Sodium; Triclonide; Triflumidate; Zidometacin; Zomepirac Sodium.

Anti-allergic agents include, but are not limited to, anti-histamines, steroids, and prostaglandin inducers. Anti-histamines are compounds which counteract histamine released by mast cells or basophils. These compounds are well known in the art and commonly used for the treatment of allergy. Anti-histamines include, but are not limited to, loratidine, cetirizine, buclizine, ceterizine analogues, fexofenadine, terfenadine, desloratadine, norastemizole, epinastine, ebastine, ebastine, astemizole, levocabastine, azelastine, tranilast, terfenadine, mizolastine, betatastine, CS 560, and HSR 609. Prostaglandin inducers are compounds which induce prostaglandin activity. Prostaglandins function by regulating smooth muscle relaxation. Prostaglandin inducers include, but are not limited to, S-5751.

Steroids include, but are not limited to, beclomethasone, fluticasone, tramcinolone, budesonide, corticosteroids and budesonide.

Corticosteroids include, but are not limited to, beclomethasome dipropionate, budesonide, flunisolide, fluticaosone, propionate, and triamcinoone acetonide.

Systemic corticosteroids include, but are not limited to, methylprednisolone, prednisolone and prednisone.

Commonly used allergy drugs which are currently in development or on the market are shown in Table 2.

Table 2

Allergy Drugs in Development or on the Market		
MARKETER	*BRAND NAME (GENERIC NAME)*	*MECHANISM*
Schering-Plough	Claritin + Claritin D (loratidine)	Anti-histamine
	Vancenase (beclomethasone)	Steroid
UCB	Reactine (cetirizine)(US) Zyrtec (cetirizine)(ex US)	Anti-histamine
	Longifene (buclizine)	Anti-histamine
	UCB 28754 (ceterizine alalogue)	Anti-histamine
Glaxo	Beconase (beclomethasone)	Steroid
	Flonase (fluticasone)	Steroid
Aventis	Allegra (fexofenadine)	Anti-histamine
	Seldane (terfenadine)	Anti-histamine
Pfizer	Reactine (cetirizine) (US) Zyrtec/Reactine (cetirizine)(ex US) (both licensed from UCB)	Anti-histamine
Sepracor	Allegra (fexofenadine)	Anti-histamine
	Desloratadine (lic to Schering-Plough)	Anti-histamine
	Cetirizine (-) (lic to UCB)	Anti-histamine
	Norastemizole (option to J&J not exercised, 10-17-99)	Anti-histamine
B. Ingelheim	Alesion (epinastine)	Anti-histamine
Aventis	Kestin (ebastine) (US) Bastel (ebastine) (Eu/Ger)	Anti-histamine
	Nasacort (tramcinolone)	Steroid
Johnson & Johnson	Hismanol (astemizole)	Anti-histamine
	Livostin/Livocarb (levocabastine)	Anti-histamine
AstraZeneca	Rhinocort (budesonide) (Astra)	Steroid
Merck	Rhinocort (budesonide)	Steroid
Eisai	Azeptin (azelastine)	Anti-histamine
Kissei	Rizaben (tranilast)	Anti-histamine
Shionogi	Triludan (terfenadine)	Anti-histamine
	S-5751	Prostaglandin inducer
Schwarz	Zolim (mizolastine)	Anti-histamine
Daiichi	Zyrtec (cetirizine)	Anti-histamine
Tanabe Seiyaku	Talion/TAU-284 (betatastine)	Anti-histamine
Sankyo**	CS 560 (Hypersensitizaion therapy for cedar pollen allergy)	Other
Asta Medica	Azelastine-MDPI (azelastine)	Anti-histamine
BASF	HSR 609	Anti-histamine
SR Pharma	SRL 172	Immunomodulation
Peptide Therapeutics	Allergy vaccine (allergy (hayfever, anaphylaxis, atopic asthma)	Downregulates specific IgE
	Tolerizing peptide vaccine (rye grass peptide (T cell epitope))	Immuno-suppressant
Coley Pharmaceutical Group	CpG DNA	Immunomodulation
Genetech	Anti-IgE	Down-regulator of IgE
SR Pharma	SRL 172	Immunomodulation

Anti-allergic agents also include asthma medicaments. Asthma medicaments include, but are not limited, PDE-4 inhibitors, bronchodilator/beta-2 agonists, K+ channel openers, VLA-4 antagonists, neurokin antagonists, TXA2 synthesis inhibitors, xanthanines, arachidonic acid antagonists, 5 lipoxygenase inhibitors, thromboxin A2 receptor antagonists, thromboxane A2 antagonists, inhibitor of 5-lipox activation proteins, and protease inhibitors.

In certain embodiments, the non-fugetactic therapeutic agents are immunosuppressants. Such immunosuppressants include: Azathioprine; Azathioprine Sodium; Cyclosporine; Daltroban; Gusperimus Trihydrochloride; Sirolimus; Tacrolimus.

Non-anti-fugetactic agents are also anti-cancer agents. Anti-cancer agents include: Acivicin; Aclarubicin; Acodazole Hydrochloride; Acronine; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametantrone Acetate; Aminoglutethimide; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperlin; Azacitidine; Azetepa; Azotomycin; Batimastat; Benzodepa; Bicalutamide; Bisantrene Hydrochloride; Bisnafide Dimesylate; Bizelesin; Bleomycin Sulfate; Brequinar Sodium; Bropirimine; Busulfan; Cactinomycin; Calusterone; Caracemide; Carbetimer; Carboplatin; Carmustine; Carubicin Hydrochloride; Carzelesin; Cedefingol; Chlorambucil; Cirolemycin; Cisplatin; Cladribine; Crisnatol Mesylate; Cyclophosphamide; Cytarabine; Dacarbazine; Dactinomycin; Daunorubicin Hydrochloride; Decitabine; Dexormaplatin; Dezaguanine; Dezaguanine Mesylate; Diaziquone; Docetaxel; Doxorubicin; Doxorubicin Hydrochloride; Droloxifene; Droloxifene Citrate; Dromostanolone Propionate; Duazomycin; Edatrexate; Eflornithine Hydrochloride; Elsamitrucin; Enloplatin; Enpromate; Epipropidine; Epirubicin Hydrochloride; Erbulozole; Esorubicin Hydrochloride; Estramustine; Estramustine Phosphate Sodium; Etanidazole; Etoposide; Etoposide Phosphate; Etoprine; Fadrozole Hydrochloride; Fazarabine; Fenretinide; Floxuridine; Fludarabine Phosphate; Fluorouracil; Flurocitabine; Fosquidone; Fostriecin Sodium; Gemcitabine; Gemcitabine Hydrochloride; Hydroxyurea; Idarubicin Hydrochloride; Ifosfamide; Ilmofosine; Interferon Alfa-2a; Interferon Alfa-2b; Interferon Alfa-n1; Interferon Alfa-n3; Interferon Beta-I a; Interferon Gamma-I b; Iproplatin; Irinotecan Hydrochloride; Lanreotide Acetate; Letrozole; Leuprolide Acetate; Liarozole Hydrochloride; Lometrexol Sodium; Lomustine; Losoxantrone Hydrochloride;

Masoprocol; Maytansine; Mechlorethamine Hydrochloride; Megestrol Acetate; Melengestrol Acetate; Melphalan; Menogaril; Mercaptopurine; Methotrexate; Methotrexate Sodium; Metoprine; Meturedepa; Mitindomide; Mitocarcin; Mitocromin; Mitogillin; Mitomalcin; Mitomycin; Mitosper; Mitotane; Mitoxantrone Hydrochloride; Mycophenolic Acid; Nocodazole; Nogalamycin; Ormaplatin; Oxisuran; Paclitaxel; Pegaspargase; Peliomycin; Pentamustine; Peplomycin Sulfate; Perfosfamide; Pipobroman; Piposulfan; Piroxantrone Hydrochloride; Plicamycin; Plomestane; Podofilox; Porfimer Sodium; Porfiromycin; Prednimustine; Procarbazine Hydrochloride; Puromycin; Puromycin Hydrochloride; Pyrazofurin; Riboprine; Rogletimide; Safingol; Safingol Hydrochloride; Semustine; Simtrazene; Sparfosate Sodium; Sparsomycin; Spirogermanium Hydrochloride; Spiromustine; Spiroplatin; Streptonigrin; Streptozocin; Sulofenur; Talisomycin; Taxotere; Tecogalan Sodium; Tegafur; Teloxantrone Hydrochloride; Temoporfin; Teniposide; Teroxirone; Testolactone; Thiamiprine; Thioguanine; Thiotepa; Tiazofurin; Tirapazamine; Topotecan Hydrochloride; Toremifene Citrate; Trestolone Acetate; Triciribine Phosphate; Trimetrexate; Trimetrexate Glucuronate; Triptorelin; Tubulozole Hydrochloride; Uracil Mustard; Uredepa; Vapreotide; Verteporfin; Vinblastine Sulfate; Vincristine Sulfate; Vindesine; Vindesine Sulfate; Vinepidine Sulfate; Vinglycinate Sulfate; Vinleurosine Sulfate; Vinorelbine Tartrate; Vinrosidine Sulfate; Vinzolidine Sulfate; Vorozole; Zeniplatin; Zinostatin; Zorubicin Hydrochloride.

Non-fugetactic therapeutic agents also include cell-growth potentiating agents which stimulate growth of a cell and includes growth factors such as PDGF, EGF, FGF, TGF, NGF, CNTF, and GDNF.

The methods provided can also include the administration of a second agent that influences cell migration. These agents may be either chemoattracting or chemorepelling depending on the embodiment. In a preferred embodiment, such agents include cytokines. "Cytokine" is a generic term for non-antibody soluble proteins which are released from one cell subpopulation and which act as intercellular mediators, for example, in the generation or regulation of an immune response. See *Human Cytokines: Handbook for Basic & Clinical Research* (Aggrawal, et al. eds., Blackwell Scientific, Boston, Mass. 1991) (which is hereby incorporated by reference in its entirety for all purposes). Cytokines include, e.g.,

interleukins IL-1 through IL-15, tumor necrosis factors α & β, interferons α, β, and γ, tumor growth factor beta (TGF-β), colony stimulating factor (CSF) and granulocyte monocyte colony stimulating factor (GM-CSF). The action of each cytokine on its target cell is mediated through binding to a cell surface receptor. Cytokines share many properties of hormones, but are distinct from classical hormones in that *in vivo*, they generally act locally on neighboring cells within a tissue. The activities of cytokines range from promoting cell growth (e.g., IL-2, IL-4, and IL-7), and arresting growth (IL-10, tumor necrosis factor and TGF-β), to inducing viral resistance (IFN α, β, and γ). See Fundamental Immunology (Paul ed., Raven Press, 2nd ed. 1989); Encyclopedia of Immunology, (Roitt ed., Academic Press 1992) (which are hereby incorporated by reference in their entirety for all purposes). In certain embodiments, the cytokine is a cytokine with chemoattractant and/or chemokinetic properties. Examples of such cytokines include: PAF, N-formylated peptides, C5a, LTB_4, LXA_4, chemokines: CXC, IL-8, GCP-2, GROα, GROβ, GROγ, ENA-78, NAP-2, IP-10, MIG, I-TAC, SDF-1α, BCA-1, PF4, Bolekine, MIP-1α, MIP-1β, RANTES, HCC-1, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5 (mouse only), Leukotactin-1 (HCC-2, MIP-5), Eotaxin, Eotaxin-2 (MPIF2), Eotaxin-3 (TSC), MDC, TARC, SLC (Exodus-2, 6CKine), MIP-3α (LARC, Exodus-1), ELC (MIP-3β), I-309, DC-CK1 (PARC, AMAC-1), TECK, CTAK, MPIF1 (MIP-3), MIP-5 (HCC-2), HCC-4 (NCC-4), MIP-1γ (mouse only), C-10 (mouse only); C: Lymphotactin; CX_3C: Fracktelkine (Neurotactin). Most preferably, the cytokine is a member of the Cys-X-Cys family of chemokines (chemokines that bind to the CXCR-4 receptor). Preferred such agents of the invention include SDF-1α, SDF-1β, and met-SDF-1β. In further preferred embodiments, such agents include other CXCR-4 receptor ligands. CXCR-4 ligands include, but are not limited to, $HIV\text{-}1_{IIIB}$ gp120, small molecules T134 and MD3100, and/or T22 ([Tyr5,12,Lys7]-polyphemusin II) (Heveker et al., Curr Biol, 1998, 8:369-76).

The compositions, as described above, are administered in effective amounts. The effective amount will depend upon the mode of administration, the particular condition being treated and the desired outcome. It will also depend upon the stage of the condition, the age and physical condition of the subject, the nature of

concurrent therapy, if any, and like factors well known to the medical practitioner. For therapeutic applications, it is that amount sufficient to achieve a medically desirable result. In some cases this is a local (site-specific) reduction of inflammation. In other cases, it is inhibition of tumor growth and/or metastasis. In some instances, an effective amount of a fugetactic agent is the amount that promotes the migration of cells away from a specific site in a subject. In other instances an effective amount of an anti-fugetactic agent is an amount that inhibits the migration of cells away from a specific site in a subject.

Generally, doses of active compounds of the present invention would be from about 0.01 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from 50-500 mg/kg will be suitable.

A variety of administration routes are available. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. The compositions of the invention can be administered systemically or locally. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Oral administration will be preferred for prophylactic treatment because of the convenience to the patient as well as the dosing schedule. When peptides are used therapeutically, in certain embodiments a desirable route of administration is by pulmonary aerosol. Techniques for preparing aerosol delivery systems containing peptides are well known to those of skill in the art. Generally, such systems should utilize components which will not significantly impair the biological properties of the antibodies, such as the paratope binding capacity (see, for example, Sciarra and Cutie, "Aerosols," in Remington's Pharmaceutical Sciences, 18th edition, 1990, pp 1694-1712; incorporated by reference). Those of skill in the art can readily determine the various parameters and conditions for producing antibody or peptide aerosols without resort to undue experimentation.

In some embodiments the fugetactic agents or anti-fugetactic agents can be targeted to a specific site. Targeting methods are known by those of ordinary skill in

the art. For example, the agents of the invention can be conjugated to a targeting molecule. As another example, a HSP can be conjugated to a targeting molecule. Targeting molecules include, but are not limited to, molecules such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell. Targeting molecules, therefore include antibodies specific for tumor antigens.

Tumor-antigens include Melan-A/MART-1, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)--C017-1A/GA733, Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin and γ-catenin, p120ctn, $gp100^{Pmel117}$, PRAME, NY-ESO-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1, CT-7, cdc27, adenomatous polyposis coli protein (APC), fodrin, P1A, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papilloma virus proteins, Smad family of tumor antigens, Imp-1, EBV-encoded nuclear antigen (EBNA)-1, and c-erbB-2.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active agent. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water,

alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compounds.

In certain embodiments, the fugetactic agents or anti-fugetactic agents of the invention are delivered directly to the site where the fugetactic agent is needed (e.g. at a site of inflammation; the joints in the case of a subject with rheumatoid arthritis, the blood vessels of an atherosclerotic organ, etc.). For example, this can be accomplished by attaching an isolated fugetactic molecule (nucleic acid or polypeptide) to the surface of a balloon catheter; inserting the catheter into the subject until the balloon portion is located at the site of inflammation, e.g. an atherosclerotic vessel, and inflating the balloon to contact the balloon surface with the vessel wall at the site of the occlusion. In this manner, the compositions can be targeted locally to particular inflammatory sites to modulate immune cell migration to these sites. In another example the local administration involves an implantable pump to the site in need of such treatment. Preferred pumps are as described above. In a further example, when the treatment of an abscess is involved, the fugetactic agent may be delivered topically, e.g., in an ointment/dermal formulation. Optionally, the fugetactic molecules of the invention are delivered in combination with a non-fugetactic molecule (e.g., anti-inflammatory, immunosuppressant, anticancer, etc.).

In a preferred embodiment of the invention, the isolated fugetactic agents of the invention are administered to a subject in combination with a balloon angioplasty procedure. A balloon angioplasty procedure involves inserting a catheter having a deflated balloon into an artery. The deflated balloon is positioned

in proximity to the atherosclerotic plaque and the site of inflammation, and is inflated such that the plaque is compressed against the arterial wall. As a result, the layer of endothelial cells on the surface of the artery is disrupted, thereby exposing the underlying vascular smooth muscle cells. The isolated fugetactic molecule is attached to the balloon angioplasty catheter in a manner which permits release of the isolated fugetactic molecule at the site of the atherosclerotic plaque and the site of inflammation. The isolated fugetactic molecule may be attached to the balloon angioplasty catheter in accordance with standard procedures known in the art. For example, the isolated fugetactic molecule may be stored in a compartment of the balloon angioplasty catheter until the balloon is inflated, at which point it is released into the local environment. Alternatively, the isolated fugetactic molecule may be impregnated on the balloon surface, such that it contacts the cells of the arterial wall as the balloon is inflated. The fugetactic molecule also may be delivered in a perforated balloon catheter such as those disclosed in Flugelman, et al., *Circulation*, v. 85, p. 1110-1117 (1992). See, also, e.g., published PCT Patent Application WO 95/23161, for an exemplary procedure for attaching a therapeutic protein to a balloon angioplasty catheter. This procedure can be modified using no more that routine experimentation to attach a therapeutic nucleic acid to the balloon angioplasty catheter.

The invention in other aspects includes pharmaceutical compositions of fugetactic agents and anti-fugetactic agents.

The fugetactic agents, anti-fugetactic agents, or fragments thereof may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptably

compositions. Such preparations may routinely contain salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

Fugetactic agents (nucleic acids or polypeptides) may be produced recombinantly. Recombinantly produced fugetactic agents such as HSPs, include chimeric proteins comprising a fusion of a HSP protein with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, sequence specific nucleic acid binding, enhancing stability of the HSP polypeptide under assay conditions, providing a detectable moiety, such as green fluorescent protein, or providing a targeting moiety. A polypeptide fused to a fugetactic polypeptide or fragment may also provide means of readily detecting the fusion protein, e.g., by immunological recognition or by fluorescent labeling.

Various techniques may be employed for introducing nucleic acids of the invention (sense and anti-sense, dominant negative) into cells, depending on whether the nucleic acids are introduced *in vitro* or *in vivo* in a host. Such techniques include transfection of nucleic acid-$CaPO_4$ precipitates, transfection of nucleic acids associated with DEAE, transfection with a retrovirus including the nucleic acid of interest, liposome mediated transfection, and the like. For certain uses, it is preferred to target the nucleic acid to particular cells. In such instances, a vehicle used for delivering a nucleic acid of the invention into a cell (e.g., a retrovirus, or other virus; a liposome) can also have a targeting molecule attached thereto. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell can be bound to or incorporated within the nucleic acid delivery vehicle. For example, where liposomes are employed to deliver the nucleic acids of the invention, proteins which

bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake. Such proteins include capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like. Polymeric delivery systems also have been used successfully to deliver nucleic acids into cells, as is known by those skilled in the art. Such systems even permit oral delivery of nucleic acids.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the fugetactic agent, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono- di- and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which the anti-inflammatory agent is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,667,014, 4,748,034 and 5,239,660 and (b) difusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,832,253, and 3,854,480.

A preferred delivery system of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vessels which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vessels (LUV), which range in size from 0.2 - 4.0 μm can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within

the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, (1981) 6:77). In order for a liposome to be an efficient gene transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the gene of interest at high efficiency with retention of biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.

Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis, G. in *Trends in Biotechnology*, (1985) 3:235-241.

In one important embodiment, the preferred vehicle is a biocompatible microparticle or implant that is suitable for implantation into the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. PCT/US/03307 (Publication No. WO 95/24929, entitled "Polymeric Gene Delivery System"). PCT/US/0307 describes a biocompatible, preferably biodegradable polymeric matrix for containing an exogenous gene under the control of an appropriate promoter. The polymeric matrix is used to achieve sustained release of the exogenous gene in the patient. In accordance with the instant invention, the fugetactic agents described herein are encapsulated or dispersed within the biocompatible, preferably biodegradable polymeric matrix disclosed in PCT/US/03307.

The polymeric matrix preferably is in the form of a microparticle such as a microsphere (wherein a fugetactic agent is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein a fugetactic agent is stored in the core of a polymeric shell). Other forms of the polymeric matrix for containing a fugetactic agent include films, coatings, gels, implants, and stents. The size and composition

of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix is introduced. The size of the polymeric matrix further is selected according to the method of delivery which is to be used. Preferably when an aerosol route is used the polymeric matrix and fugetactic agent are encompassed in a surfactant vehicle. The polymeric matrix composition can be selected to have both favorable degradation rates and also to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time.

In another important embodiment the delivery system is a biocompatible microsphere that is suitable for local, site-specific delivery. Such microspheres are disclosed in Chickering et al., *Biotech. And Bioeng.*, (1996) 52:96-101 and Mathiowitz et al., *Nature*, (1997) 386:.410-414.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver the fugetactic agents of the invention to the subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. Synthetic polymers are preferred. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multivalent ions or other polymers.

In general, fugetactic agents or anti-fugetactic agents are delivered using a bioerodible implant by way of diffusion, or more preferably, by degradation of the polymeric matrix. Exemplary synthetic polymers which can be used to form the biodegradable delivery system include: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terepthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, poly-vinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate,

cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl alcohols), polyvinyl acetate, poly vinyl chloride, polystyrene, polyvinylpyrrolidone, and polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, poly(butic acid), poly(valeric acid), and poly(lactide-cocaprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water *in vivo,* by surface or bulk erosion.

Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof.

Bioadhesive polymers of particular interest include bioerodible hydrogels described by H.S. Sawhney, C.P. Pathak and J.A. Hubell in *Macromolecules*, (1993) 26:581-587, the teachings of which are incorporated herein, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate).

In addition, important embodiments of the invention include pump-based hardware delivery systems, some of which are adapted for implantation. Such implantable pumps include controlled-release microchips. A preferred controlled-release microchip is described in Santini, JT Jr., et al., *Nature*, 1999, 397:335-338, the contents of which are expressly incorporated herein by reference.

Use of a long-term sustained release implant may be particularly suitable for treatment of chronic conditions. Long-term release, are used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Examples

Materials And Methods

Cell Culture and Production of Conditioned Media

The EL4 cell line (ATCC, Manassas, VA) was maintained in culture in Isocove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (I10), 50 units/ml Penicillin and 50 μg/ml Streptomycin and 292 μg/ml L-Glutamine (Mediatech; Herndon, VA). EL4 24-hour conditioned media (EL4CM24) was produced by sub-culturing the EL4 cells in hybridoma serum-free media (HSF) (Invitrogen; Carlsbad, CA) supplemented with 292 μg/ml L-Glutamine (Mediatech; Herndon, VA) for three days. The cells were then transferred into fresh HSF media and cultured at an initial density of 1 x 10^6 cells/ml for 24 hours at 37°C in a 5% CO_2 humidified incubator. The cell culture supernatant was collected after 24 hours and was filtered through a 0.2 μm pore size filter before use in *in vitro* and *in vivo* assays.

Preparation of Enriched Murine T-cells

C57BL/6 mice (4 to 8 weeks old) (Jackson Laboratories; Bar Harbor ME) were euthanized, and dissection of superficial cervical, axillary, brachial and inguinal lymph nodes was performed under sterile conditions. Dissected lymph nodes were then physically disaggregated and a cell suspension generated. The cell

suspension was then enriched for T cells by depletion of non-T cells using MACS Pan T cell isolation kit for mouse leukocytes (Miltenyi Biotec; Auburn, CA). This method typically resulted in $CD3^+$ purity greater than 95% as demonstrated by flow cytometry.

Transmigration Assays

Quantitative transmigration assays were carried out as described previously [18]. In brief, ChemoTx chemotaxis 30 µl well plates with 3 µm pore size filters (Neuro Probe; Gaithersburg, MD) were used. 20,000 murine T-cells were loaded in the upper chamber of the ChemoTx system and subjected to positive, negative and uniform gradients of EL4CM24 in a standard checkerboard analysis of cell migration. The peak concentrations used in each gradient were 1 in 2, 1 in 10, and 1 in 100 dilutions of EL4CM24 with I0.5 media; Isocove's supplemented with 0.5% fetal calf serum, 50 units/ml Penicillin, 50 µg/ml Streptomycin and 292 µg/ml L-Glutamine (Mediatech; Herndon, VA). The number of cells migrating in response to the given gradient was judged to be the total number of cells in the lower chamber of the ChemoTx system after a 90 minute incubation at 37°C, in a humidified 5% CO_2 incubator. The chemotactic index was calculated as the quotient of the average number of cells migrating in response to EL4CM24 and the average number of cells migrating in response to media alone. All experiments were performed in triplicate with duplicate wells for each gradient condition.

Heat Inactivation, Proteinase K Digestion, Heat Shock and Use of Specific Inhibitors

EL4CM24 was heat inactivated at 42°C for 1 hour prior to being used in the transmigration assays described above. Viability of the cells was observed to be greater than 90%. EL4CM24 was incubated with hydrated proteinase K agarose (Sigma-Aldrich; St. Louis, MO) at 1 mg/ml for 1 hour at 37°C in a 5% CO_2 humidified incubator then filtered through a 0.2 µm pore-size filter prior to being used in the transmigration and intraperitoneal assays. Murine T-cells were incubated with pertussis toxin (Sigma-Aldrich; St. Louis, MO) at 100ng/ml for 1 hour at 37 °C in a 5% CO_2 humidified incubator, prior to being used in the transmigration assays. EL4CM24 was incubated with radiciol (Sigma-Aldrich; St. Louis, MO) for two

hours at room temperature at concentrations 0.1 μg/ml and 7.3 μg/ml. The transmigration was then carried out using the EL4CM24 mixture. As a control, transmigration assays were also carried out using gradients of HSF media supplemented with 7.3 μg/ml of radicol or using EL4CM24-radicicol mixtures from which excess radiciol had been removed using 5 kDa size exclusion Centricon Ultrafree filters (Millipore; Billerica, MA). EL4 cells in HSF were also exposed to Geldanamycin at 1.2 μM or 200 nM prior to use of the CM in transmigration assays. EL4 cells in HSF were also exposed to Brefeldin A at 10 μg/ml overnight for a full 24 hours prior to use of the EL4CM24 in transmigration assays. Greater than 65% viability was observed after the Brefeldin A treatment.

Ion Exchange Chromatography

Fractions of EL4CM24 were eluted from DEAE columns (Amersham Biosciences; Piscataway, NJ) using 20 mM triethanolamine (Sigma-Aldrich; St. Louis, MO) 0.25 M NaCl buffer at pH 7.5. Eluted fractions were desalted using PD-10 desalting columns (Amersham Biosciences; Piscataway, NJ), then concentrated using 5 kDa size exclusion Centricon Ultrafree filters (Millipore; Billerica, MA) prior to being used in the transmigration assays. Fractions of EL4CM24 were eluted at NaCl concentrations of 0.25 M, 0.5 M, 0.75 M, 1.0 M and 2.0 M. Eluted fractions were desalted prior to use in transmigration assays.

SDS PAGE

7.5 % acrylamide gels were run according to the method of Laemmli, under denaturing conditions. EL4CM24 and fractions eluted by ion exchange chromatography were prepared for SDS PAGE by boiling with Laemmli buffer and 5% β-mercaptoethanol for three minutes. Silver staining of gels was achieved using the Silver Stain Plus kit (Bio-Rad; Hercules, CA). Sequencing of protein bands from silver stained gels was completed at the Proteomics Mass Spectrometry Lab at University of Massachusetts Medical Center using standard technologies. Further information is readily available regarding mass spectrometry analysis, including MS-Fit and MS-Tag searching on the following website: http://prospector.ucsf.edu/. Briefly, MS-Fit is a "peptide mass fingerprinting tool" that analyzes mass spectrometry data and attempts to "fit" the data to a protein sequence. These protein

sequences can be from any known sequence and commonly are from existing databases. MS-Tag searching follows a similar methodology but attempts to match the ionic nature of the protein sequences.

Intraperitoneal Injection Of Conditioned Media And Analysis Of Lymphocyte Infiltration

BALB/c-DO11 mice (kindly provided by Dr. Iacomini, Massachusetts General Hospital) were primed by subcutaneous injection with100 µl of ovalbumin (ICN Biomedicals; Costa Mesa, CA) dissolved in Freund's complete adjuvant (Pierce Biotechnology; Rockford, IL) at a concentration 1 mg/ml. Three days later, mice were injected intraperitoneally with 250 µl of ovalbumin dissolved in sterile water at concentration a 0.4 mg/ml. 24 hours later, experimental mice were injected intraperitoneally with 250 µl of EL4CM24, while control mice were injected intraperitoneally with 250 µl of HSF media or protease treated EL4CM24. 24 hours later, cells were extracted from the intraperitoneal space by lavage with 5 ml of cold phosphate buffered saline (Mediatech; Herndon, VA) and aspiration with a 10 ml syringe and 21-gauge needle. Peritoneal lavage cells were counted on a hemocytometer and then immuno-stained for anti- CD3, CD4, CD8 and TCR-kj-OVA lymphocyte expression (BD BioSciences; San Jose, CA) and anti-CD4 (Caltag Laboratories; Burlingame, CA) with antibodies labelled with Percp and PE (phytoerythrin) known fluorophor labels. T-cells expressing CD3+, CD3+CD4+, CD3+CD8+, CD3+ TCR-kj-OVA were then determined by FACS analysis.

Results

EL4 Conditioned Media Repels Murine Lymphocytes In Vitro

EL4CM24 was generated from EL4 cells cultured in HSF as described above. The viability of cells in culture for 24 hours in HSF was always >95%. This is significant in that it demonstrates that there was no significant apoptosis or necrosis of the cells in culture. In transmigration assays using negative gradients of 1 in 2, 1 in 10, and 1 in 100 dilutions of EL4 24-hour conditioned media (EL4CM24) migration away from conditioned media was significant compared to

migration away from media alone, and decreased with increasing concentration of EL4CM24.

The mean migratory index of cells migrating in response to the 1 in 100, 1 in 10, and 1 in 2 dilutions of EL4CM24 was 382.50±50.55, 325.50±60.96, 163.83±35.42, respectively, representing chemotactic indices of 5.20±0.47, 4.67±0.89, 2.39±0.55. The average numbers of cells migrating in each gradient of EL4CM24 compared to the average number of cells migrating in media alone was statistically significant in each of the three concentrations of EL4CM24 tested (Fig. 2). Thus, factors in ELCM24, when presented in a negative gradient, repel T cells in vitro.

Repulsion of Murine Lymphocytes by EL4 Conditioned Media is Reduced By Heat Inactivation and Proteinase K Digestion

In transmigration assays using negative gradients of heat inactivated or proteinase K digested EL4 24-hour conditioned media, the level of migration of lymphocytes was significantly reduced by proteinase K digestion but not by heat inactivation. The mean number of cells migrating in response to the 1 in 100, 1 in 10, and 1 in 2 dilutions of heat inactivated EL4CM24 was 161.00±28.10, 230.50±41.50, 76.50±19.50, respectively, representing chemotactic indices of 2.01±0.34, 2.76±0.10, 0.91±0.07. The average numbers of cells migrating in each gradient of heat inactivated EL4CM24 compared to the average number of cells migrating in untreated EL4CM24 was only statistically significant in the highest of the three concentrations of EL4CM24 tested (Fig. 3).

The mean number of cells migrating in response to the 1 in 100, 1 in 10, and 1 in 2 dilutions of proteinase K digested EL4CM24 was 110.00±25.00, 118.00±18.10, 36.50±1.50, respectively, representing chemotactic indices of 2.50±0.00, 2.93±0.63, 0.87±0.09. The average numbers of cells migrating in each gradient of proteinase K digested EL4CM24 compared to the average number of cells migrating in untreated EL4CM24 was statistically significant in two of the three concentrations of EL4CM24 tested (Fig. 3). These results confirmed that the factors having activity in EL4CM24 are proteins.

Repulsion of Murine Lymphocytes by EL4 Conditioned Media is Reduced by Specific Inhibitors

In transmigration assays using negative gradients of EL4 24-hour conditioned media with pertussis toxin treated murine lymphocytes, the level of migration of lymphocytes was significantly reduced. The mean number of pertussis toxin treated cells migrating in response to the 1 in 100, 1 in 10, and 1 in 2 dilutions of EL4CM24 was 37.00±4.00, 26.50±0.50, 20.50±4.50, respectively, representing chemotactic indices of 3.65±0.52, 2.57±0.18, 1.93±0.09. The average numbers of pertussis toxin treated cells migrating in each gradient of EL4CM24 compared to the average number of untreated cells migrating in EL4CM24 was statistically significant in all of the three concentrations of EL4CM24 tested (Fig. 4). The use of pertussis toxin, which inhibits G-protein coupled migration by preventing the release of GDP from the $G_{\alpha i}$ subunit, indicated that the migration induced by EL4CM24 is coupled to signaling via a G-protein pathway and implies the receptors for the active proteins in the EL4CM24 are G-protein coupled.

In transmigration assays using negative gradients of radicicol treated EL4 24-hour conditioned media, the level of migration of lymphocytes was not significantly reduced. The mean number of cells migrating in response to the 1 in 100, 1 in 10, and 1 in 2 dilutions of radicicol treated EL4CM24 was 191.83±32.38, 172.83±33.12, 84.67±18.55, respectively, representing chemotactic indices of 3.49±0.78, 3.60±1.21, 2.08±0.79. The average numbers of cells migrating in each gradient of radicicol treated EL4CM24 compared to the average number of cells migrating in untreated EL4CM24 was statistically not significant in any of the three concentrations of EL4CM24 tested (Fig. 4). This in combination with the sequencing of a prominent doublet of protein bands from EL4 conditioned media, points to the presence of copious amounts of HSP90α/β as radicicol is a known inhibitor of HSP90. Although the overall level of migration of lymphocytes was reduced by the use of radicicol, the highest level of migration seen with radicicol treated EL4 conditioned media was still significant compared to media alone. This low level of inhibition may be a function of the concentration of radiciol used, or may indicate that HSP90 works in concert with some other fugetactic protein or proteins in the conditioned media to augment their effect.

The results using Geldanamycin treated EL4 24-hour conditioned media in the transmigration assays in response to the 1 in 100, 1 in 10, and 1 in 2 dilutions is also provided in Fig. 4.

Fractionation of EL4 Conditioned Media Reveals Candidate Proteins Which Elicit Repulsion of Murine Lymphocytes

Elution of fractions of 24-hour EL4 conditioned media from DEAE columns using 0.25M NaCl resulted in two fractions associated with the repulsion of murine lymphocytes when tested in transmigration assays. Sequencing of component proteins from the fractions with the highest activity identified demonstrated that the fugetactic activity was associated with the presence of a 65 kDa, 84 kDa, 86 kDa, 94 kDa and 110 kDa proteins in the EL4CM24. Results from the MS-Fit and MS-Tag searches are provided in Figs. 12-14. Specifically, the results depicted in these figures provide the identity of the proteins (i.e., HSPs and L-plastin) that contained peptide sequences that are homologous to the component proteins from the EL4CM24 fractions.

EL4 CM Repels Immune Cells In Vivo

A T-cell infiltrate and subsequent allergic immune reaction was established in the intraperitoneal cavity of BALB/C DO11 mice using established techniques (3). EL4CM24, HSF or protease treated EL4CM24 was instilled into the inflamed peritoneal cavity to establish how these agents would affect T-cell infiltration into the anatomic space. EL4CM24 alone significantly reduced ($p < 0.05$) the infiltration of all T-cell subtypes into the peritoneal cavity and in particular those T-cells bearing the OVA specific TCR (Fig. 5). Protease treated EL4CM24 and HSF alone did not have a significant effect on T-cell infiltration into the peritoneal cavity.

Immunoprecipitation of HSP90α from CM with a monoclonal antibody against the protein in conditioned medium from EL-4 cells led to a four-fold reduction in the fugetactic effect of the CM on T-cells infiltrating the peritoneal cavity following immune challenge in vivo.

EL4 CM Repels Immune Cells In Vivo

Although the fugetactic protein is likely a secreted protein, heat shock did not lead to its overexpression. In transmigration assays using negative gradients of heat shocked (at 42° C) EL4 24-hour conditioned media, the level of migration of lymphocytes was not significantly increased by heat shock (Fig. 8).

Fig. 8 also provides the results from the treatment of the EL4 24-hour conditioned media with Brefeldin A.

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